D 2014

UNDERLYING THE ROLE OF P-CADHERIN IN BREAST CANCER CELL METABOLISM

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TESE DE DOUTORAMENTO APRESENTADA
AO INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
DA UNIVERSIDADE DO PORTO EM
PATOLOGIA E GENÉTICA MOLECULAR

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Tese de Candidatura ao grau de Doutor em Patologia e Genética Molecular submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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UNDERLYING THE ROLE OF P-CADHERIN

IN BREAST CANCER CELL METABOLISM

A todos os que travam esta luta diariamente.

Financial Support:

PhD Fellowship: SFRH/BD/69353/2010

Fundação para a Ciência e Tecnologia (FCT)

AGRADECIMENTOS

Chegar até aqui, entre muitas outras coisas, implica a escrita de umas palavras de agradecimento. Pensei que seria mais fácil escolher as palavras que traduzem o sentimento que tenho e as memórias que guardo bem no cantinho do meu coração. Mas a verdade é que, mesmo as palavras mais bem escolhidas nunca se irão aproximar de tudo o que sinto por todos aqueles que me deram a mão e fizeram este percurso comigo. O meu mais sentido e profundo obrigada a todos.

Joana, foi longa a nossa viagem. Tantas conquistas e tantas "coisas boas" que me permitiste aprender contigo. O teu sentido de missão, a forma como me ensinaste a levantar a cabeça e a seguir em frente. A tua honestidade intelectual, a tua genuinidade. Mas mais do que ciência, levo comigo um pedaço de ti, da tua coragem, determinação e a forma como devolvias tudo isto embrulhado num pacote de carinho. E por isso, especialmente a ti, o meu muito obrigada!

Dr. Fernando, lembro-me da primeira reunião da "Mama", que eu acabei por assistir por acaso, no dia em que cheguei ao Ipatimup. Para mim, aquela reunião foi um resumo do que eu sabia que iam ser os próximos tempos: o início da minha carreira, vindo a fazer parte daquele grupo que falava tão bem sobre cancro da mama. Foi, de verdade, um prazer partilhar estes anos com o Dr. Fernando, ouvi-lo atentamente, absorver e aprender tudo o que dizia com a mais intensa paixão pela ciência e medicina. Foi com essa mesma paixão que me contagiou e me incentivou a querer sempre aprender mais e melhor durante todos estes anos, até hoje. A verdade é que aprendi consigo que nada se faz sem paixão e que a ciência acontece com um brilho nos olhos. Apesar de todas as ameaças de me esganar e me por em hipoxia *in vivo*, o meu mais sincero obrigada!

À Raquel Seruca, a matriarca do IPATIMUP, o meu MUITO OBRIGADA. Obrigada pela liderança, pelo sentido de missão partilhada e acima de tudo por me arrancar sorrisos e borboletas da cabeça. É por isso, e pela capacidade de nunca deixar de

“surpreender” que tenho orgulho em seguir o seu caminho, o caminho que já é de tantos no Cancer Genetics.
Ao Professor Sobrinho Simões, o meu obrigado por cuidar tão bem da nossa casa e por querer sempre mais para nós. É para mim uma honra fazer parte de um projecto tão corajoso e arrojado como o Ipatimup.

À Fátima Baltazar, co-orientadora deste trabalho, a quem agradeço todo o apoio prestado, a disponibilidade e a sempre tão pronta resposta!

A todos os co-autores dos artigos incluídos nesta dissertação agradeço a colaboração e o espírito de equipa.

A todos os que me ofereceram as condições estruturais para que pudesse trilhar o meu caminho pela ciência: ao IPATIMUP; ao Instituto de Ciências Biomédicas Abel Salazar (ICBAS); à Universidade do Porto e à Fundação para a Ciência e Tecnologia (FCT).

To Goran Landberg, I wish to thank you for receiving me in your lab at Paterson Institute for Cancer Research (PICR). It was a great opportunity for me as a researcher to be at Paterson and to absorb all the knowledge that the institute offers. I wish also to make a special “Thank you!” to Hannah Harrison for the encouragement before and during my time at Manchester, for being always available to help me in the lab and to discuss the results with me. Thank you Hannah, for being so tenderly there to me. Still in Manchester, e acho que posso escrever em Português, um obrigada muito especial ao Ricardo, à Amy, ao Bruno, à Àsun e à Yatsi. Obrigada por não só terem aberto as portas das vossas casas mas por o terem feito tão carinhosamente. Obrigada pela tentativa constante de me fazerem sentir em casa, ainda que tão longe. Gui, um beijinho e um obrigada por tudo!

To Françoise Bono, I want to thank you for receiving me at SANOFI and for giving me the opportunity to be a part of your great team. The time that I spent in SANOFI gave

me not only the possibility to work with “my so adored” SEAHORSE, to realize how much cancer metabolism fascinates me, but also to get to know all the great people I met at SANOFI. To Nathalie Alet, “*merci”* for being always available for talking, teaching, discussing and laughing with me, all at the same time and with the enthusiasm that I will always remember from you. It was a real pleasure to meet you. A special “*merci”* also to Isabelle for the support in the lab but especially for doing it “avec plaisir”. Still, and because I will always remember you all with a big smile for the everyday love and affection, for all the “*bonjours*”, for trying to teach me some French words and expressions, for lending me your bicycles and for the “*Friday steak haché*”, for these and many other loving details that I brought from Toulouse, “*Merci Bien”*!

Ao meu tão querido grupo da Mama! À Ana Sofia, obrigada por me receberes e me acolheres no laboratório com paciência e carinho, e por durante todos estes anos me apoiares nas minhas tentativas de fazer ciência! Ao André Albergaria, por me “picares os miolos” desde que cheguei ao Ipatimup; Ao André Vieira por me chamares “riqueza” a toda a hora; à minha Dizinha, por estares sempre ao meu lado de braços bem abertos; à Madalena, por tantos minutos de partilha; à Ritinha, por ser tão fácil descrever-te em duas palavras: “In” e “suportável”; sem esquecer as meninas novas, a Filipa e a Joana! E ainda aos que por cá passaram e deixaram um pouco de si naquele instituto como a Nair, a Maria Rita, a Daniela, o Nuno Bernardes, o Zé Luís, a Sílvia e a Fernanda! Obrigada a todos pela partilha e cumplicidade criada ao longo destes anos nas reuniões e no laboratório! Obrigada, meninos da Mama, pela presença em dias não produtivos, em vitórias e pelos nossos momentos de loucura.

Obrigada aos patologistas envolvidos neste trabalho, pelo esforço bem sucedido na criação das séries de cancro da mama bem como na análise dos resultados: ao Dr. Veronese, à Dra. Daniella Vieira, à Dra. Rozany Dufloth, ao Dr. Luiz Zeferino, ao Dr. Vitor Carneiro, ao Dr. René Gerhard, à Dra. Fernanda Milanezi e ao Dr. Fernando Schmitt.

Ao meu tão querido Cancer Genetics, a todos os que fazem parte e aos que já nos "abandonaram"! Obrigada por todos os bons momentos, incluindo os de loucura saudável que nos fazia aguentar dias intermináveis no laboratório. Obrigada por me aguentarem a cantar no laboratório e nos corredores, pelos lanches do CG, pelos abraços colectivos e por tudo o que fez destes anos, anos memoráveis! À minha querida Dina Leitão, à Regina e à Sara Ricardo, obrigada por me introduzirem no mundo da Anatomia Patológica e por tantos anos de partilha. Quero ainda deixar aqui um beijinho e um "muito obrigada" à Catarina Leitão, pela ajuda que me deu nas tardes passadas no citómetro no IBMC e por fazer o que faz com tanto empenho!

À minha companhia constante e fora de horas! Ritinha, ainda que me mantenha fiel ao "insuportavelzinha", foste essencial para mim desde que chegaste! Obrigada pelo incentivo: "Estás a dormir?!"; pela força: "Não sei o que andas aqui a fazer!", por acreditares: "Eu é que vou fazer o teu doutoramento todo!" e por tantas outras palavras de ajuda! Bom, a verdade é que eu agradeço do fundo do coração toda a tua ajuda de todas as formas, perto, longe, fora de horas, no laboratório e nos nossos *brainstormings*. Obrigada pela tua tão especial presença na minha vida. Pati, obrigada pela paciência e disponibilidade constante! Dizinha, obrigada pelo teu sorriso enternecedor e amizade constante! Daniela, de aluna a amiga, gosto muito de ti, minha querida! Inesinha, pequena, obrigada por tomares conta da fome! Mafaldinha, foi um prazer, minha querida! Joana Carvalho, obrigada pela tua atenção! Mónic, mesmo colada ao telemóvel, obrigada por tudo! Ricardo, miúdo!!!!! E ainda que por pouco tempo, um beijinho enorme para Catarina Salgado, Estefânia e Ana Justino! Pizinha, um beijinho com saudades da tua energia!
Ao meu amigo e companheiro Daniel, por todos estes anos de partilha, cumplicidade e amizade, por estares sempre lá, com ou sem um chocolate ou gelado para acompanhar uma boa conversa, o meu mais sincero obrigada.

À Zezinha, à Cátia, ao Sr. Mário, ao Sr. Oliveira e Sr. Mendes, um obrigada especial. Mais do que peças fundamentais do Ipatimup, foram e são para mim uma boa parte do meu carinho por este instituto!

A todos aqueles que passaram pela copa do Ipatimup e fizeram dos meus almoços momentos especiais. Tantas risadas que ficaram marcadas naquelas paredes. Obrigada!

Aos meus ursos…um obrigada muito especial por fazerem “tanto e tão bem” parte da minha vida e desta etapa! Cada um à sua maneira (para não dizer “cada tolo com a sua panca”), e todos juntos, ajudaram-me a manter a minha sanidade a par da minha loucura! Por isso, pelo carinho, pelo apoio, companhia, por “valas comuns”, por cartas atrasadas e inesperadas, pela ânsia do fim do dia e do fim-de-semana, por tudo e tanto que vocês sabem ser…o meu muito obrigada!

À Maria, à Li e ao meu Patrão! Bom, este não é fácil! Descrever momentos, é pouco para tantos que são! Qualifica-los…não existem palavras “tão grandes” que se aproximem do calor do nosso abraço de urso! Só com fotos antigas me apercebo quanto tempo passou! Só assim me vem à memória um momento, um pedido de ajuda, uma ajuda sem pedido, um olhar sem palavras, tantas outras palavras para tão pouco, muita dor de barriga e lágrimas de tanto rir, a sensação de “vai ficar tudo bem” e tantos outros sentimentos que fizeram de vocês minha propriedade: a minha Maria, a minha Licas e o meu Patrão! Obrigada pela caminhada na ciência, perante toda a desilusão e fascínio, mas essencialmente obrigada pela mais pura e altruísta amizade, da vossa Bázinha sai um: “Lob ius”!

Aos meus padrinhos…por sempre me incentivarem com palavras de encorajamento, com elogios e até com algum gozo! Por acreditarem sempre que eu seria capaz de tudo, por me fazerem sentir sempre e mo dizerem na hora certa que estão ao meu lado, e principalmente por me fazerem querer ser o que eu sou aos vossos olhos! O meu mais sincero e eterno obrigada. À minha Eduardinha, que por crescermos juntas, fez de mim muito do que eu sou e consigo hoje, por me ter dado os meus *babes* José e António, e por me fazer sempre acreditar que eu ia conseguir! Aos meus *babes*, que enchem a “Ti Bebi” de amor e coragem! E claro, ao Gilinho e ao Tiago, por fazerem parte da minha família e o fazerem tão bem!

Aos meus animais! Sem voz, ensinaram a dedicar-me ao que quer que faça, de corpo e alma. Sem saberem, tantas vezes renovaram a minha energia, esta que precisei para chegar até aqui! Sem pedirem, o colinho que vos dava sempre me confortou mais a mim do que a vocês. À minha Lisa e ao meu Fox por continuarem a aquecer o meu coração; ao meu Succy, por te saber ao meu lado ao acordar de manhã; à Cuca, Griskas, Branquinha, Lucky e Milu! A todos os meus meninos do abrigo! Obrigada do fundo do meu coração.

A ti, Lena, tu que és tanto! Tu, que por seres tu, sempre foste um dos meus maiores pontos de referência, o meu porto seguro, para onde sempre soube que podia ir e sempre fui! Tu que me dizias que tudo ia correr bem, que é “normal”, que sempre me fizeste acreditar que eu ia alcançar com sucesso todos os meus objectivos, mesmo quando eu tinha a certeza que ia falhar redondamente. Tu que me ensinaste a sentir e amar como eu sinto e amo! Por todos estes anos, por todos os Sábados repletos de amor incondicional, por todos os momentos de angústia e de felicidade. Por tudo e por tanto, o meu mais sincero e profundo obrigada!

Aos meus pais! Aos meus pais eu agradeço quem sou, tudo o que me deram e o que não me deram! Chegar até aqui é, sem dúvida alguma, fruto das oportunidades que vocês me proporcionaram, das que eu abracei porque me incentivaram, das decisões que eu tomei com o vosso apoio, do empurrãozinho que eu ia precisando e ainda do travão que, muitas vezes, também precisei! O meu para sempre obrigada! Obrigada por acreditarem em mim, por me apoiarem e por me incentivarem em todas as fases da minha vida, por serem meus pais e por estarem sempre lá! E a ti, Nequinha, por à nossa maneira, nos sabermos presentes e constantes para o que quer que a vida nos traga!

A todos, que tanto e tão bem me acompanharam nesta longa caminhada: MUITO OBRIGADA! E...tudo de bom!

DIRETIVAS LEGAIS

No cumprimento do disposto no Decreto-Lei no 230/2009, declara-se que a autora desta dissertação participou ativamente na conceção e na execução do trabalho experimental que estiveram na origem dos resultados apresentados, bem como na sua interpretação e na redação dos respetivos manuscritos.

O seguinte artigo científico original faz parte integrante desta Tese de Doutoramento:

1. Sousa B, Ribeiro AS, Nobre AR, Lopes N, Martins D, Pinheiro C, Vieira AF, Albergaria A, Gerhard R, Schmitt F, Baltazar F, Paredes J. The basal epithelial marker P-cadherin associates with breast cancer cell populations harboring a glycolytic and acid-resistant phenotype. BMCCancer. 2014; 14(1):734 (IF=3.319)

O artigo de revisão seguinte, embora não sendo parte integrante desta dissertação, foi usado na Introdução deste trabalho:

2. Albergaria A, Ribeiro AS, Vieira AF, **Sousa B**, Nobre AR, Seruca R, Schmitt F and Paredes J. P-cadherin role in normal breast development and cancer. Int J Dev Biol. 2011; 55(7-9): 811-822 (IF=2.856)

Adicionalmente, a autora desta dissertação contribuiu para as seguintes publicações que incluem partes da introdução desta tese:

3. Ribeiro AS, Albergaria A, **Sousa B**, Correia AL, Bracke M, Seruca R, Schmitt FC and Paredes J. Extracellular cleavage and shedding of P-cadherin: a mechanism underlying the invasive behaviour of breast cancer cells. Oncogene. 2010; 29(3):392-402 (IF=7.135)

4. Albergaria A, Ribeiro AS, Pinho S, Milanezi M, Carneiro V, **Sousa B,** Sousa S, Oliveira C, Machado JC, Seruca R, Paredes J, and Schmitt F. ICI 182,780 induces P-

cadherin overexpression in breast cancer cells through chromatin remodelling at the promoter level: a role for C/EBP{beta} in CDH3 gene activation. Hum Mol Genet. 2010; 13, 2554-66 (IF=7,386)

5. Sousa B, Paredes J, Milanezi F, Lopes N, Martins D, Dufloth R, Vieira D, Albergaria A, Veronese L, Carneiro V, Carvalho S, Costa JL, Zeferino L and Schmitt F. P-cadherin, vimentin and CK14 for identification of basal-like phenotype in breast carcinomas: an immunohistochemical study. Histol Histopathol. 2010; 25(8):963-974 (IF=2,404)

6. Pinheiro C, Albergaria A, Paredes J, **Sousa B**, Dufloth R, Vieira D, Schmitt F and Baltazar F. Monocarboxylate transporter 1 is up-regulated in basal-like breast carcinoma. Histopathol. 2010; 56(7):860-867 (IF=3.855)

7. Lopes N, **Sousa B**, Martins D, Gomes M, Vieira D, Veronese LA, Milanezi F, Paredes J, Costa JL and Schmitt F. Alterations in Vitamin D signalling and metabolic pathways in breast cancer progression: a study of VDR, CYP27B1 and CYP24A1 expression in benign and malignant breast lesions. BMC Cancer. 2010; 10:483 (IF=2.736)

8. Martins D, **Sousa B**, Lopes N, Gomes M, Veronese L, Albergaria A, Paredes J and Schmitt F. Molecular phenotypes of matched in situ and invasive components of breast carcinomas. Hum Pathol. 2011; 42, 1438-46 (IF=2.998)

9. Pinheiro C, **Sousa B**, Albergaria A, Paredes J, Dufloth R, Vieira D, Schmitt F and Baltazar F. GLUT1 and CAIX expression profiles in breast cancer correlate with adverse prognostic factors and MCT1 overexpression. Histol Histopathol. 2011; 26(10):1279-1286. (IF=2.502)

10. Lopes N, Carvalho J, Duraes C, **Sousa B**, Gomes M, Costa JL, Oliveira C, Paredes J and Schmitt F. 1Alpha,25-dihydroxyvitamin D3 induces de novo E-

cadherin expression in triple-negative breast cancer cells by CDH1-promoter demethylation. Anticancer Res. 2012; 32(1):249-257. (IF=1.725)

11. Moniz S, Martinho O, Pinto F, **Sousa B**, Loureiro C, Oliveira MJ, Moita LF, Honavar M, Pinheiro C, Pires M, Lopes JM, Jones C, Costello JF, Paredes J, Reis RM and Jordan P. Loss of WNK2 expression by promoter gene methylation occurs in adult gliomas and triggers Rac1-mediated tumour cell invasiveness. Hum Mol Genet. 2013; 22(1):84-95. (IF=7.692)

12. Ribeiro AS, **Sousa B**, Carreto L, Mendes N, Nobre AR, Ricardo S, Albergaria A, Cameselle-Teijeiro JF, Gerhard R, Söderberg O, Seruca R, Santos MA, Schmitt F and Paredes J. P-cadherin functional role is dependent on E-cadherin cellular context: a proof of concept using the breast cancer model. J Pathol. 2013; 229(5):705-18 (IF=7.585)

13. Albergaria A, Resende C, Nobre AR, Ribeiro AS, **Sousa B**, Machado JC, Seruca R, Paredes J and Schmitt F. CCAAT/enhancer binding protein beta (C/EBPbeta) isoforms as transcriptional regulators of the pro-invasive CDH3/P-cadherin gene in human breast cancer cells. PLoS One. 2013; 8(2):e55749. (IF=3.730)

14. Martins D, Beca FF, **Sousa B**, Baltazar F, Paredes J and Schmitt F. Loss of caveolin-1 and gain of MCT4 expression in the tumor stroma: key events in the progression from an in situ to an invasive breast carcinoma. Cell Cycle. 2013; 12(16):2684-2690. (IF=5.321)

15. Vieira AF, Ribeiro AS, Dionisio MR, **Sousa B**, Nobre AR, Albergaria A, Santiago-Gomez A, Mendes N, Gerhard R, Schmitt F, Clarke RB and Paredes J. P-cadherin signals through the laminin receptor alpha6beta4 integrin to induce stem cell and invasive properties in basal-like breast cancer cells. Oncotarget. 2014; 5(3):679-692. (IF=6.627)

TABLE OF CONTENTS

ABBREVIATION LIST

AMF Autocrine motility factor

AMPK Adenosine monophosphate-activated protein kinase

AUC Area under curve

ctn Catenin

BLBC Basal-like breast cancer

BRCA1 Breast cancer susceptibility gene 1

BSA Bovine serum albumin

CBD Catenin binding domain

CAIX Carbonic anhydrase IX

Cdc42 Cell division cycle 42 Rho-family GTPase

CDH1 Cadherin 1 or E-cadherin gene

CDH3 Cadherin 3 or P-cadherin gene

cDNA Complementary DNA

CK Cytokeratin

CSC Cancer stem cells

CXCR4 C-X-C chemokine receptor type 4

DAPI 4,6-diamidino-2-phenylindole

DNA Desoxyribonucleic acid

E-cadherin Epithelial cadherin

ECAR Extracellular acidification rate

ECM Extracellular matrix

EGFR Epidermal growth factor receptor

EMMPRIN Extracellular matrix metalloproteinase inducer

EMT Epithelial to mesenchymal transition

ER Oestrogen receptor

ESC Embryonic stem cells

ETC Electron transport chain

FACS Fluorescence activated cell sorting

FBS Fetal bovine serum

FdG Fluorodeoxyglucose

GEP Gene expression profile

GLUT Glucose transporter

GSC Glioma stem cells

GTPase Guanine tri-phosphatase

HER2 Receptor tyrosine-protein kinase erbB-2

HIF Hypoxia-inducible factor

HMEC human mammary epithelial cells

HR Hormone receptors

HRE Hypoxia responsive elements

HSC Hematopoietic stem cell

IBC Inflammatory breast cancer

iPSC Induced-pluripotent stem cells

JMD Juxtamembrane domain

LDH Lactate dehidrogenase

LOX Lysyl oxidase

MCT Monocarboxylate transporter

MFE Mammosphere formation efficiency

MMP Matrix metalloproteinases

mRNA Messenger RNA

NADH Reduced nicotinamide adenine dinucleotide

NAD^+ Nicotinamide adenine dinucleotide

NAF Nipple aspirate fluid

NBT Nitroblue tetrazolium

NO Nitric oxide

NSC Neural stem cell

OCR Oxygen consumption rate

OXPHOS Oxidative phosphorylation

PBS Phosphate buffered saline

P-cadherin Placental cadherin

PDH Pyruvate dehydrogenase

PET Positron-emission tomography

PgR Progesterone receptor

PKM2 Pyruvate dehydrogenase kinase M2

PPP Pentose phosphate pathway

Rac1 Ras-related C3 botulinum toxin substrate 1

Rho Ras homologue gene

RNA Ribonucleic acid

ROS Reactive oxygen species

sP-cad Soluble P-cadherin

SOD Superoxide dismutase

TCA Tricarboxylic acid

TGF-α Transforming growth factor alpha

TIC Tumour-initiating cell

TMA Tissue microarray

TNBC Triple-negative breast cancer

uPAR Urokinase plasminogen Activator Receptor

VEGF Vascular endothelial growth factor

VHL Von Hippel Lindau

ABSTRACT

Abstract

Constitutive upregulation of glycolysis is likely to be a cellular adaption to hypoxic conditions, being recently recognized as a hallmark of cancer. Interestingly, it has been shown that cancer stem cells (CSC), as well as normal stem cells, use preferentially glycolysis over oxidative phosphorylation as their main source of energy, exhibiting increased adaptation to oxidative stress, with enhanced antioxidant protective systems and low reactive oxygen species (ROS) content. These properties provide cells with increased invasive and metastatic properties, as well as increased ability to escape to oxidative stress-induced anoikis. In breast cancer, these CSC, which are known to persist in the human body after therapy due to their unique metabolic properties, are responsible for promoting relapses and metastasis.

P-cadherin, a calcium dependent cell-cell adhesion molecule encoded by the *CDH3* gene, is a protein whose expression is highly associated with undifferentiated cells of adult epithelial tissues, including the normal mammary gland, as well as with poorly differentiated carcinomas. In breast cancer, P-cadherin is aberrantly expressed in high-grade tumours with basal-like phenotype, being a well-established indicator of poor patient prognosis. Moreover, P-cadherin expression promotes breast cancer cell invasion, partially through the secretion of matrix metalloproteinases (MMPs), such as MMP1 and MMP2, and induces cell-extracellular matrix (ECM) attachment and alterations in the actin cytoskeleton. Importantly, P-cadherin is also known to promote stem-like properties in breast cancer cells, such as tumourigenic capacity and anoikis-resistance, allowing cells to survive in anchorage-independent conditions. The expression of this protein confers radiation-resistance to breast cancer cells and, upon apoptotic stimuli, decreased P-cadherin expression increases breast cancer cell death in a caspase-dependent mechanism.

In this work, we demonstrate, for the first time, that aberrant P-cadherin expression is associated with the hypoxic/glycolytic and acid resistant phenotype in invasive breast carcinomas, represented by a panel of markers including HIF-1α (hypoxia-inducible factor 1α), GLUT1 (glucose transporter 1), CAIX (carbonic anhydrase IX), MCT1 (monocarboxylate transporter 1) and CD147. We also show that P-cadherin expression is modulated by hypoxia in a time dependent manner, that HIF-1α

stabilization increases membrane P-cadherin expression and that P-cadherin-enriched cancer cell population shows increased GLUT1 and CAIX expression. These populations also comprise high mammosphere forming efficiency, suggesting that P-cadherin overexpressing breast cancer cells are more likely to exhibit increased glycolysis and to survive to metabolic-driven pH alterations.
We were still able to demonstrate that P-cadherin expression is responsible for oxidative phosphorylation (OXPHOS) suppression in BLBC (basal-like breast cancer) cells, due to the observation that downregulation of its expression induces a metabolic shift towards OXPHOS, with concomitant increase of ATP production. Furthermore, we observed that oxidative stress is modulated by P-cadherin expression in breast cancer cells, since its overexpression was associated with low ROS levels, probably due to the induced upregulation of superoxide dismutases 1 and 2 (SOD1 and 2) antioxidant systems.

Taken together, these results led us to postulate that HIF-1α might be stabilizing membrane P-cadherin expression in breast CSC, or even selecting P-cadherin-enriched breast CSC populations in the hypoxic niche. In turn, P-cadherin expression is inducing a metabolic reprogramming and antioxidant response of these cells, which might be responsible for tumour aggressiveness, as well as for their ability to survive and resist, compared to the neighbour low P-cadherin expressing cells. In this case, P-cadherin is mediating the survival of aggressive cells thought to be resistant to chemo and radiotherapy, being responsible for tumour relapses and metastasis in breast cancer patients.

RESUMO

O aumento constitutivo do metabolismo glicolítico é, provavelmente, um mecanismo de adaptação celular a condições de hipóxia, sendo actualmente reconhecido como um dos "*Hallmarks of Cancer*". Curiosamente, tem sido demonstrado que as células estaminais do cancro, bem como células estaminais normais, utilizam preferencialmente a glicólise como principal fonte de energia, apresentando assim uma maior adaptação ao stress oxidativo, com sistemas de protecção antioxidante aumentados e baixos níveis de espécies reactivas de oxigénio. Estas propriedades aumentam a capacidade invasiva e metastática das células tumorais, uma vez que conferem uma maior aptidão para estas escaparem à morte induzida por stress oxidativo (*anoikis*). Assim, devido às suas propriedades metabólicas, foi demonstrado que as células estaminais de cancro da mama permanecem no corpo humano após terapias oncológicas, sendo responsáveis por recidivas e formação de metástases em doentes com este tipo de cancro.

A caderina-P é uma molécula de adesão célula-célula dependente de cálcio, codificada pelo gene *CDH3*, cuja expressão se encontra associada a células indiferenciadas de tecidos epiteliais adultos, como a glândula mamária, bem como a carcinomas pouco diferenciados. Em cancro da mama, a caderina-P encontra-se sobre-expressa em tumores de alto grau histológico e com fenótipo basal, sendo um indicador de mau prognóstico. Sabe-se também que a expressão de caderina-P promove a invasão de células de cancro da mama, parcialmente através da secreção de metaloproteinases de matriz (MMPs), como MMP1 e MMP2, promovendo ainda a ligação célula-matriz extracelular e induzindo alterações no citoesqueleto de actina. A caderina-P é conhecida pela promoção de propriedades estaminais em células de cancro da mama, tais como a capacidade tumorigénica e resistência à *anoikis*, permitindo que estas células sobrevivam em condições independentes da adesão à matriz. A expressão desta proteína confere também resistência à radiação e, mediante estímulos apoptóticos, a diminuição da sua expressão induz a morte de células de cancro da mama, através de um mecanismo dependente de caspases.

Neste trabalho, demonstrámos pela primeira vez, que a expressão aberrante de caderina-P se encontra associada ao fenótipo hipóxico, glicolítico e de resistência à acidose em carcinomas invasivos da mama, através da avaliação da expressão de

um painel de marcadores como HIF-1α, GLUT1, CAIX, MCT1 e CD147. Observámos que a expressão de caderina-P é modulada por hipóxia, de uma forma dependente do tempo, que a estabilização de HIF-1α induz aumento de expressão de caderina-P na membrana celular, e ainda que as populações de células com expressão aumentada de caderina-P se encontram igualmente enriquecidas na expressão de GLUT1 e CAIX. Estas populações mostram também uma capacidade aumentada de formação de mamosferas, sugerindo que as células de cancro da mama com expressão aberrante de caderina-P apresentam uma capacidade glicolítica aumentada e uma maior capacidade de sobreviver a variações de pH induzidas por alterações metabólicas.

Neste trabalho, mostrámos ainda que a caderina-P é responsável pela supressão da fosforilação oxidativa em células de cancro da mama, bem como pela adaptação ao stress oxidativo, uma vez que células com expressão aberrante desta proteína apresentam níveis reduzidos de espécies reactivas de oxigénio, provavelmente associados à indução de sistemas antioxidantes, como SOD1 e SOD2.

Em suma, estes resultados sugerem que o factor de transcrição HIF-1α estabiliza a expressão de caderina-P na membrana das células de cancro da mama, ou que selecciona populações de células com aumento de expressão desta molécula de adesão em nichos hipóxicos. Por sua vez, a caderina-P induz uma reprogramação metabólica e uma adaptação ao stress oxidativo nestas células, podendo ser responsável pelo aumento da agressividade tumoral, bem como pelo aumento da capacidade de sobrevivência das células enriquecidas na expressão de caderina-P.
Em conclusão, acreditamos que a caderina-P medeie a sobrevivência de células agressivas de cancro da mama, com resistência a quimio e radioterapia, responsáveis pelo aparecimento de recidivas e metástases em doentes com cancro da mama.

THESIS OUTLINE

In Chapter I, a general introduction presents the current knowledge about cancer metabolism, as well as about metabolism and stemness/differentiation. It summarizes several aspects of cancer metabolism, namely the effect of tumour microenvironment in this context and the metabolic switch observed in stem cells and CSCs, with a particular focus in breast carcinogenesis and breast CSCs. Moreover, it also discloses what is currently known and described regarding the role of P-cadherin expression in cell differentiation and in breast cancer.

The rationale and aims of this Thesis are presented in Chapter II, which are in line with the background described in the introduction.

Chapter III describes the material and methods and Chapter IV encloses original data of this Thesis, published in BMC Cancer, as well as unpublished and ongoing work, which are in preparation to be published in an international peer reviewed journal.

A general discussion integrating all the results is presented in Chapter V, followed by Conclusions and Future Perspectives in Chapter VI.

CHAPTER I

Introduction

1. METABOLISM AND CANCER

The link between cancer and metabolism is not new. Early observations in the beginning of the cancer research area identified metabolic alterations as a common feature of tumours. However, these were proven to be inadequate to explain the tumourigenic process and the field of oncogene research pushed metabolism to the margins of cancer research. Recently, there is a renewed interest on tumour metabolism, since several signalling pathways affected by oncogenic alterations and tumour microenvironment were found to have a significant effect in metabolism, making “cancer metabolism” a subject highly important in cancer biology.

1.1 THE WARBURG EFFECT

In the presence of oxygen, most differentiated cells rely primarily on mitochondrial oxidative phosphorylation (OXPHOS) to generate energy for cellular processes, metabolizing glucose to carbon dioxide by oxidation of glycolytic pyruvate in the mitochondrial tricarboxylic acid (TCA) cycle (**Figure 1**). The final electron acceptor in glucose oxidation is oxygen, being an essential element in this process. This reaction produces NADH (reduced nicotinamide adenine dinucleotide), which fuels OXPHOS to maximize ATP (adenosine triphosphate) production, with minimal production of lactate. However, in anaerobic conditions, differentiated cells redirect pyruvate away from the mitochondria and produce large amounts of lactate, with the production of lower levels of energy. Most cancer cells and proliferating tissues also produce great quantities of lactate, regardless of the availability of oxygen, being their metabolism often referred as “aerobic glycolysis”. This effect was originally described by Otto Warburg, which hypothesized that cancer cells present mitochondrial defects leading to impaired aerobic respiration, and subsequently rely on glycolytic metabolism for ATP production [3]. However, normal mitochondrial function was observed in cancer cells and in normal proliferating cells, suggesting the existence of an alternative explanation for aerobic glycolysis in cancer cells [4, 5].

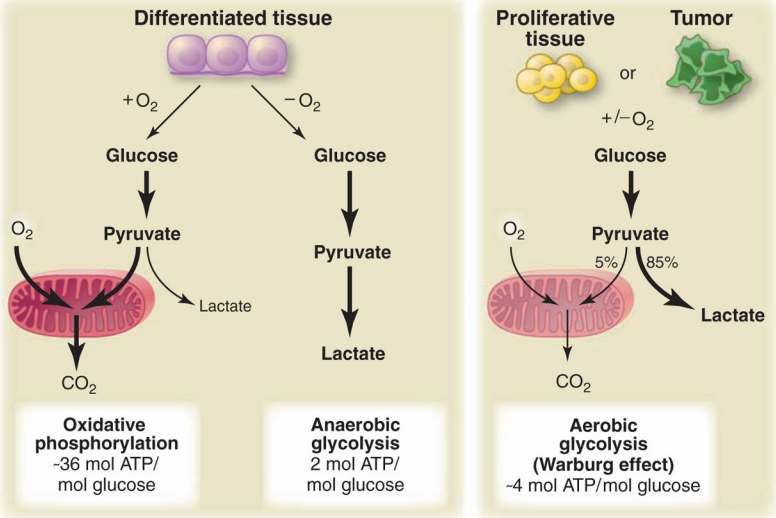


Figure 1. Representation of the differences between oxidative phosphorylation, anaerobic glycolysis and aerobic glycolysis in differentiated, cancer and proliferating cells. In the presence of oxygen, differentiated tissues metabolize glucose to pyruvate via glycolysis and then completely oxidize most of that pyruvate in the mitochondria to CO_2 during oxidative phosphorylation. When oxygen is in limiting levels, cells can redirect the pyruvate generated by glycolysis away from mitochondrial oxidative phosphorylation by generating lactate (anaerobic glycolysis). Production of lactate during anaerobic glycolysis allows glycolysis to continue (by cycling NADH back to NAD+), but results in minimal ATP production when compared with oxidative phosphorylation. Normal proliferative tissues and cancer cells tend to convert most glucose to lactate, regardless the presence of oxygen (aerobic glycolysis/Warburg effect). Adapted from Vander Heiden *et al.* [6]

Nevertheless, aerobic glycolysis is a less efficient mechanism for ATP generation comparing to oxidative phosphorylation. In proliferating cells, approximately 10% of the glucose is diverted into other biosynthetic pathways upstream of pyruvate production. The metabolism of glucose to lactate generates only 2 ATPs per molecule of glucose, whereas OXPHOS generates up to 36 ATPs after complete oxidation of one glucose molecule [6]. Furthermore, enhanced glycolysis imposes the production of lactate, which is further discarded by the cell.

These observations led to the following question: why a less efficient energetic metabolism is selected in proliferating and in cancer cells? The dynamic regulation of cell's metabolism should be taken into account in order to understand this paradox. Cell metabolism is an integration of cellular needs with external signals, such as morphogens, substrates, and oxygen, among others. Cancer cells are relatively independent from these external signals in order to maintain their uncontrolled growth, being this characteristic one of the hallmarks of cancer [7, 8]. Thus, these

cells use flexibly their metabolic processes with the adoption of distinct metabolic programs. Terminally differentiated cells adopt a catabolic metabolism for maximal energetic efficiency, providing them appropriate energetic charge to sustain their functions, as is the case of high energy-requiring phenotypes, such as cardimyocytes and neurons [9]. However, when a cell is committed to rapid and active proliferation, ATP and NADH are not the only required products, and glycolysis and TCA do not function only for compensating cellular energetic demands. In such cases, these pathways and their intermediate products are deviated to other molecular pathways, such as pentose phosphate pathway (PPP), hexosamine synthesis and serine/glycine synthesis pathways, in order to provide precursors for the synthesis of lipids, proteins, DNA and RNA (**Figure 2**) [6, 9, 10].

Thus, proliferating cells usually adopt anabolic metabolism so that energy production and the synthesis of molecular precursors necessary for cell growth and subsequent division are assured. Moreover, ATP production by glycolysis is much faster than by OXPHOS and, if enough glucose is available, sufficient amounts of energy will be produced. Furthermore, ATP levels are sensed by the cells, allowing them to adapt their metabolic processes. When increased levels of cellular ATP are present, there is a decrease in glycolytic rates by inactivation of AMPK (5' adenosine monophosphate-activated protein kinase). ATP can also act as a direct allosteric inhibitor of glycolytic enzymes. Still, the metabolic shift towards glycolysis has also been proposed as a mechanism to protect cells from increased ROS produced in the mitochondria [11, 12].

Metabolism is a dynamically regulated system, where the activity of several enzymes allows the cell to fit the requirements for proliferation energetic needs of each differentiated tissue. In normal proliferating tissues, such as in the developing embryos, signals from growth factors allow cells to utilize nutrients to match their need [13, 14]. In cancer, oncogenic pathways drive cell-autonomous nutrient uptake and program proliferative metabolism, whereas tumour suppressor pathways prevent nutrient utilization for anabolic processes. Thus, oncogenic alterations provide cells the ability to acquire nutrients and coordinately regulate metabolic pathways to support proliferation and survival [6].

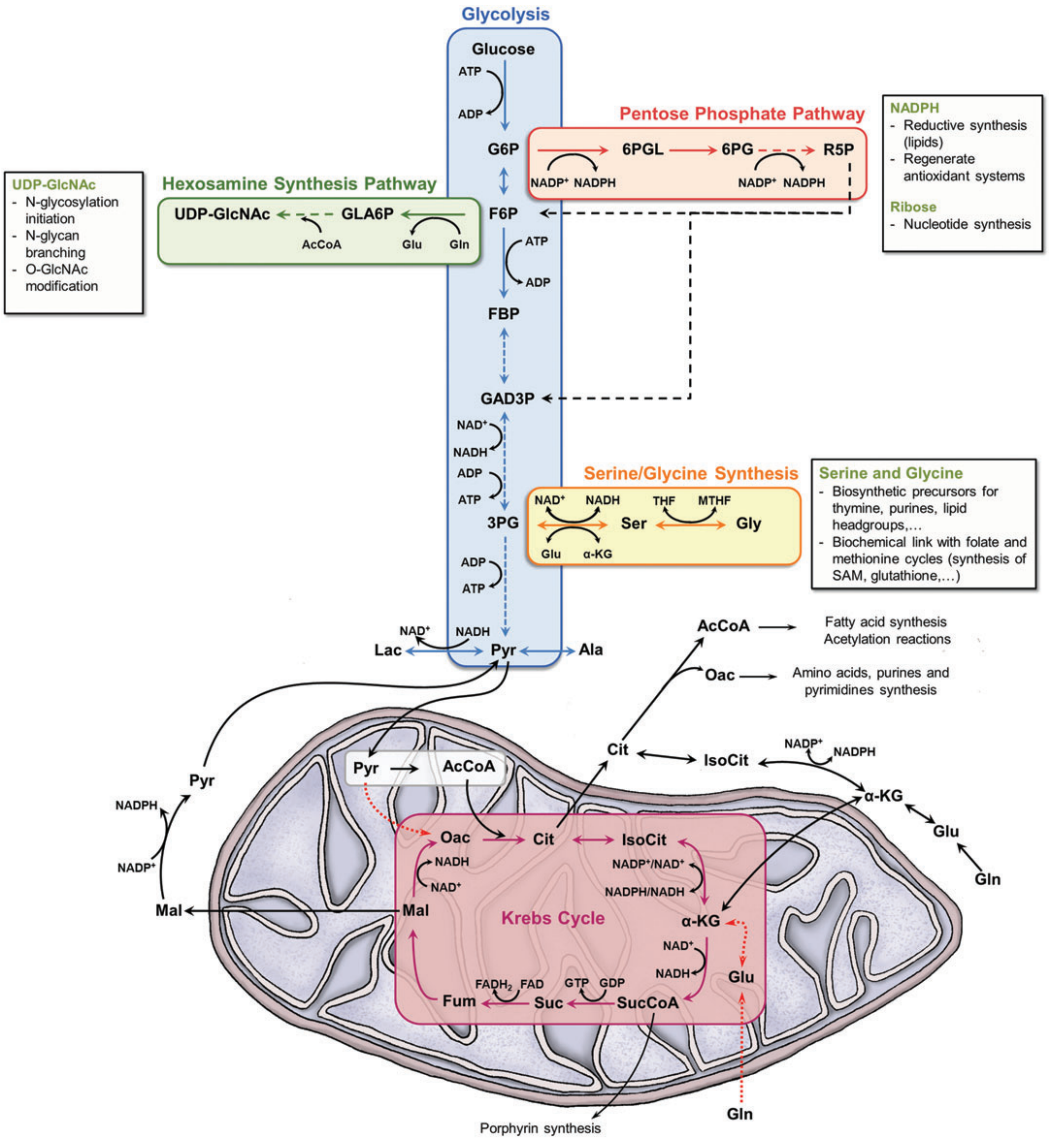


Figure 2. Metabolic pathways underlying active proliferation. The significance of some of the metabolites and intermediates resulting from the main pathways branching from glycolysis. Red dotted arrows represent anaplerotic reactions of the Krebs cycle. Abbreviations: 3PG, 3-phosphoglycerate; 6PG, 6-phosphogluconate; 6PGL, 6-phosphogluconolactone; α-KG, α-ketoglutarate; AcCoA, acetyl-co-enzyme A; Ala, alanine; Cit, citrate; FBP, fructose-1,6-biphosphate; F6P, fructose-6-phosphate; Fum, fumarate; GLA6P, glucosamine-6-phosphate; G6P, glucose-6-phosphate; Glu, glutamate; Gln, glutamine; GAD3P, glyceraldehyde-3-phosphate; Gly, glycine; IsoCit, isocitrate; Lac, lactate; Mal, malate; MTHF, methylenetetrahydrofolate; Oac, oxaloacetate; Pyr, pyruvate; R5P, ribose-5-phosphate; Ser, serine; Suc, succinate; SucCoA, succinyl-co-enzyme A; THF, tetrahydrofolate; UDP-GlcNAc, uridine diphosphate-N-acetylglucosamine. Adapted from Pereira *et al.* [9]

Interestingly, glycolysis upregulation is the basis of the clinical tumour imaging FdG PET (^{18}fluorodeoxyglucose Positron-Emission Tomography), which uses the glucose analogue tracer FdG to mimic glucose [15, 16]. This imaging technique, based on the enhanced glycolysis of tumours, allows the identification of primary and metastatic tumours in oncology patients (**Figure 3**).

Figure 3. Positron-emission tomography imaging with ^{18}fluorodeoxyglucose of a patient with lymphoma. Nodes indicated by arrows show high uptake of FdG, demonstrating that tumour cells in these nodes have high levels of FdG uptake. The bladder (yellow arrow) also has high activity, because of excretion of the radionuclide). Adapted from Gillies and Gatenby [1].

1.2 Cell-Environment Model for Carcinogenesis: The Implications of Hypoxia and Glycolysis Upregulation

Aerobic glycolysis is a near-universal observation in tumours, being a crucial component of the malignant phenotype [17] and a new hallmark of cancer [8]. Upregulation of glycolytic metabolic pathways in tumours is the result of an adaptation of cancer cells to consistent environmental pressures in pre-malignant lesions, when diffusion thought to be responsible for the development of invasive and metastatic tumour cells. Cellular traits selected by these conditions include constitutive upregulation of glycolysis and resistance to acid-induced apoptosis [1, 18]. In an attempt to explain carcinogenesis, Gatenby and Gillies proposed a model of cell-environment interactions, where hypoxic environment and acquisition of a glycolytic phenotype and acidosis resistance are implicated in cancer development and progression [1, 18]. Since blood vessels are confined to the stromal compartment,

early carcinogenesis occurs in an avascular environment. In pre-malignant lesions, increased proliferation leads to a thickening of the epithelial layer and cells become distant from their blood supply, developing in a hypoxic environment. Hypoxia then favours selection of the best adapted cells to harsh conditions of low oxygen and nutrients availability by the acquisition of a glycolytic phenotype. As a consequence of increased acidosis, tumours become acidotic, leading to the selection of cells with increased motility and invasion abilities. After the breakdown of the basement membrane, cells have access to blood and lymphatic vessels, which constitute the main routes for the spreading of cancer cells, promoting metastatic disease (**Figure 4**).

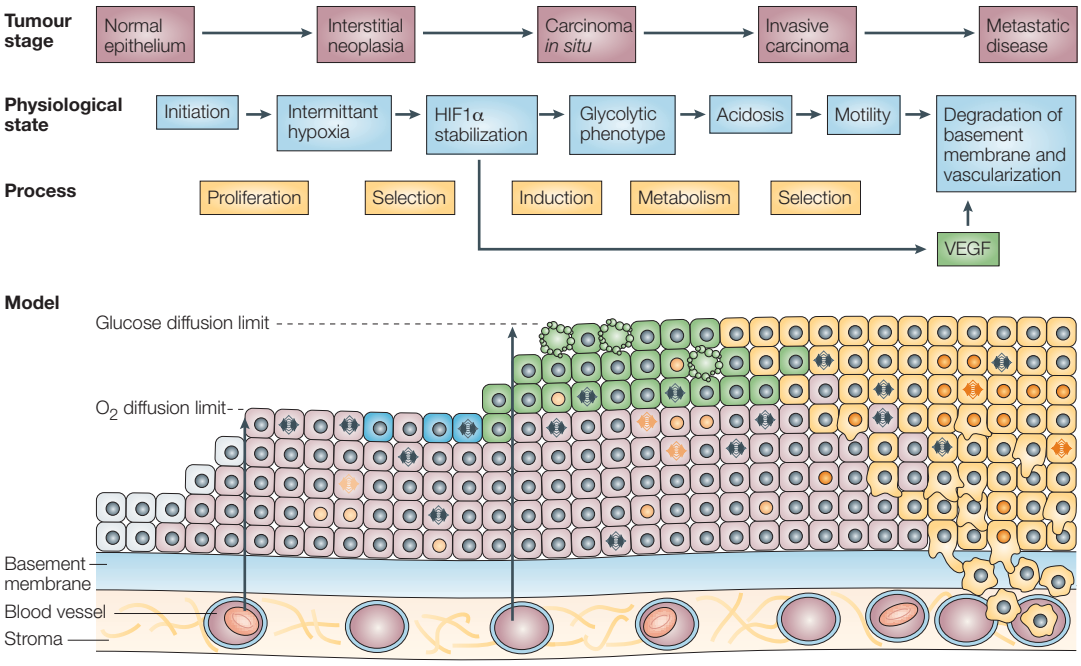


Figure 4. Model for cell–environment interactions in carcinogenesis. The stages of tumour growth and their associated physiological states, showing that progression from one stage to the next is governed by state processes. Normal epithelial cells (grey) become hyperproliferative (pink) following induction. As they reach the oxygen diffusion limit, they become hypoxic (blue), which can either lead to cell death (apoptotic cells shown with blebbing) or adaptation of a glycolytic phenotype (green), which allows cells to survive. As a consequence of glycolysis, lesions become acidotic, which selects for motile cells (yellow) that eventually breach the basement membrane. As cancer progression proceeds, the mutations in cells increase (nuclei shown as light orange for one mutation and darker oranges for more mutations). Adapted form Gatenby and Gillies [1].

Taken together, these authors propose that environmental constraints represent crucial evolutionary steps in the development of invasive cancer. With the acquisition of a phenotype with a powerful proliferative advantage through persistent aerobic glycolysis, cells modulate the local microenvironment with increased acidosis, which can be harmless to itself, but fatal to other non-adapted populations. This acidification facilitates tumour invasion, both through destruction of adjacent normal populations, as well as through acid-induced degradation of the ECM and promotion of angiogenesis [1].

1.3 Hypoxia in Breast Cancer

Several molecular mechanisms lead to constitutive upregulation of aerobic glycolysis. The key regulator of the glycolytic response is the transcription factor HIF-1α, first discovered by Semenza and Wang [19]. HIF-1α combines with HIF-1β to form a heterodimeric transcription factor that regulates the expression of several cellular processes. HIF-1α and HIF-1β are constitutively expressed in cells. However, HIF-1α expression is destabilized in the presence of O_2 by proline hydroxylation, being targeted for proteassomal degradation by the Von Hippel Lindau (VHL) ubiquitin ligase [20-22]. When it accumulate in cells, as in the case of low oxygen tensions, the HIF-1 complex binds hypoxia responsive elements (HREs; canonically CCATG) in the promoter region of target genes. The transcriptional activity of HIF-1α is known to mediate the cellular response to hypoxic stress by inducing genes involved in survival, glycolysis, angiogenesis, pH regulation, as well as haematopoiesis [23].

Hypoxic microenvironment is a feature of many solid tumours and occurs when a rapidly dividing tumour mass exceeds the existent vascular supply [24, 25]. Areas of necrosis can be observed in highly proliferative solid tumours and hypoxia has also been associated with treatment resistance, local invasion, poor clinical outcome and mortality [23, 26-28]. In addition, the undifferentiated phenotype of solid tumours, including breast cancer, strongly correlates with tumour hypoxia [29].

Interestingly, it has been widely demonstrated the effect of hypoxia, via HIF-1α, in the induction of EMT (epithelial to mesenchymal transition), invasion and migration. EMT

is characterized by the acquisition of migratory ability by the cells, including the loss of E-cadherin expression [30]. Several reports have been demonstrating the association of hypoxia with loss of E-cadherin expression in several types of cancer, including breast cancer [31]. One of the responsible mechanisms seems to involve HIF-1α induction of LOX (Lysyl Oxidase), which is highly expressed in breast cancer cells with high metastatic potential [32, 33]. LOX is able to modify ECM components [34] and is also known to activate the expression of Snail. So, HIF-1α induces LOX activation, which in turn leads to Snail activation and consequently to the repression of E-cadherin expression. The other described mechanism for hypoxia-induced E-cadherin repression involves the Notch signalling and the activity of HES1 and HEY2, leading to Slug and Snail increased expression and E-cadherin downregulation [31].

Within the wide range of genes induced by HIF, some are involved in the acquisition of invasion, migratory and proteolytic activities, namely vimentin, fibronectin, cytokeratins (CK14, 18 and 19), matrix metalloproteinase 2 (MMP2), cathepsin D and urokinase plasminogen activator receptor (uPAR) [25]. Migratory ability is also potentiated by hypoxia/HIF-1α through the induction of autocrine motility factor (AMF), receptor tyrosine kinase c-Met, TGF-α (transforming growth factor-α), as well as of cytokine receptor (CXCR4) [25, 35-37]. Still, hypoxia/HIF-1α is also implicated in the selection, expansion and maintenance of cancer cell populations with stem-like properties [38, 39].

Hypoxia has been recognized as being determinant for clinical outcomes in several human tumours, including breast cancer [40-42]. Several authors have associated hypoxia-related gene signatures with poor prognosis in breast cancer. Winter *et al.* demonstrated that hypoxia metagene of head and neck cancer (HNSCC) present a poor prognosis relevance in independent breast cancer data sets [41]. Moreover, Chi *et al.* showed that human tumours, including breast carcinomas, could be stratified according to their hypoxia response, demonstrating that breast tumours with a strong gene expression signature of response to hypoxia, present a significantly worse prognosis, correlated with breast cancer progression and metastasis [40]. These authors also demonstrated that the prognostic information of the hypoxia signature was independent of other previously reported signatures and more predictive of

outcomes than other clinical parameters used in the clinical practice [40].

HIF-1α expression is observed in 25-40% of invasive breast carcinomas and is associated to poor prognosis, short patient's survival, high proliferation and poor tumour differentiation [43-45]. Moreover, the expression of this transcription factor is a predictive marker of chemotherapy failure. Generalli *et al.* demonstrated a significant inverse correlation of HIF-1α expression before treatment and patient's disease response [43]. Moreover, Schwab *et al.* also demonstrated that HIF-1α enhances primary tumour growth and lung metastasis using a model of HIF-1α wild-type and HIF-1α-null mammary tumour epithelial cells (MTECs) [46].

Interestingly, hypoxia has been also shown to decrease ER expression in breast cancer cell lines [47], either at the transcriptional [48] or post-transcriptional levels, through proteasome dependent degradation [49-51]. Still, loss of ER expression is observed in breast tumours in hypoxic/necrotic areas of the tissue [49, 51], associating hypoxia with dedifferentiation by loss of oestrogen receptor (ER) expression.

Moreover, BRCA1-related mutation carriers, which typically develop tumours with a basal-like phenotype [52], were described as presenting high levels of HIF-1α expression, comparing with sporadic breast tumours [53]. Furthermore, Yan *et al*. proposed that the aggressive nature of BRCA1 and basal-like tumours may be explained by an enhanced hypoxic response through the altered expression of proteins involved in the O_2-dependent mechanism of HIF-1α degradation [54]. Furthermore, overexpression of HIF-1α also associates with the reduction of reactive oxygen species [55], increased radioresistance [56, 57] and protection of cells from drug induced apoptosis and senescence cancer therapy [58, 59].

1. 4 Metabolic Switch and Molecular Adaptation to Glycolysis

Constitutive upregulation of glycolysis is likely to be an adaption to hypoxia, since this condition selects cells that rely on anaerobic metabolism [1]. However, some cancer cells that do not persist in hypoxic environment seem to exhibit preferentially glycolysis over oxidative phosphorylation. Examples of these highly glycolytic cells

that reside in high oxygen levels are leukemic cells, that can be found in the bloodstream [13, 60], and lung cancer cells, that are exposed to oxygen during tumourigenesis [61, 62]. Although HIF-1α strongly links aerobic glycolysis to carcinogenesis [63], it is not entirely true that glycolytic phenotype in tumours is invariably due to hypoxia. Besides hypoxia, a wide range of other physiological and pathological factors can activate HIF system, such as growth promoters like insulin growth factor and epidermal growth factor amplification systems, together with Ras and Myc oncogenes [64]. Moreover, alterations in tumour suppressor genes, such as p53 and VHL, can also be responsible for the activation of this system [22, 23]. For example, mutation in the VHL, in which the wild-type function targets HIF-1α for degradation, are associated with constitutively high HIF-1α expression and therefore with high glucose consumption rates, as in the case of renal cell carcinomas [65]. Moreover, the observation of glycolysis upregulation in primary tumours and in metastasis also leads to the suggestion that, besides being a solution to microenvironmental growth constrains, increased glycolysis in solid tumours seems to be a hallmark of cancer [8, 17]. Thus, these evidences suggest that hypoxia may not be a major contributor in the switch to aerobic glycolysis by cancer cells.

As a requirement for the increased glucose uptake, GLUTs must be upregulated in this process. GLUT1 is the most well studied member of the GLUT family of glucose transporters, which mediates transport of glucose into the cells. It is expressed in several types of tumours such as brain, non-small cell lung and hepatocellular carcinomas [66-68]. This protein is also present in breast cancer [69], being associated with poor prognosis [70, 71]. Moreover, GLUT1 is an important stress response mediator of cancer cell survival when glucose is limited, and its role in tumour growth in *in vitro* and *in vivo* mammary mouse models was already described [72], promoting the consideration of this molecule as an attractive therapeutic target. The importance and prevalence of aerobic glycolysis in breast cancer is highlighted by the importance of glucose metabolism in breast cancer cells. In fact, several reports have demonstrated the increased glucose uptake and modulation of the expression of specific GLUTs in breast cancer cells [70, 73]. Specifically, GLUT1 expression was found to be upregulated in breast cancer cells, being associated with

higher proliferation and high histological grade [70]. Consistently, it was shown that glucose deprivation, as well as treatment with 2-deoxy-D-glucose (2-DG), induces breast cancer cell apoptosis, reinforcing their dependence on glucose metabolism [74]. In accordance with the reliable identification of primary and metastatic tumours using FdG PET, glycolytic rates are also associated with aggressiveness, either *in vitro* or *in vivo* as well as in breast cancer patients. Interestingly, higher ^{18}FDG was observed in more aggressive molecular subtypes of breast carcinomas, namely in HER2 (receptor tyrosine-protein kinase erbB-2) overexpressing and triple negative tumours [75].

However, this increased glycolysis, the so-called “metabolic switch”, results in the acidification of the intracellular space. In normal cells, prolonged exposure to acidosis might result in necrosis or apoptosis by a p53- and caspase 3-dependent mechanism [76-78]. In tumours, cells harbour alterations, such as p53 mutations, that allow them to escape acidosis-induced cell death and thus continue to proliferate. So, constitutive upregulation of glycolysis requires an additional adaptation to the negative effects of acidosis through resistance to apoptosis or upregulation of membrane transporters, responsible for cellular homeostasis (**Figure 5**) [79].

The intracellular pH homeostasis is maintained by upregulation of several H^+ transporters, leading to the acidification of the extracellular medium [1]. Cancer cells have the ability to acidify the extracellular medium by different mechanisms, such as the extrusion of lactic acid and also by proton-pump activity [80]. This acidification leads to the activation of metalloproteases and/or cathepsins [80, 81], which will be responsible for the degradation of the ECM and basement membrane, facilitating tumour invasion and metastasis [82]. Montcourrier *et al.* demonstrated that metastatic, highly invasive and hormone receptor (HR) negative breast cancer cell lines have a higher ability to acidify their extracellular medium comparing to non-invasive and non-metastatic cell lines, hormonal receptor expressing cells [80].

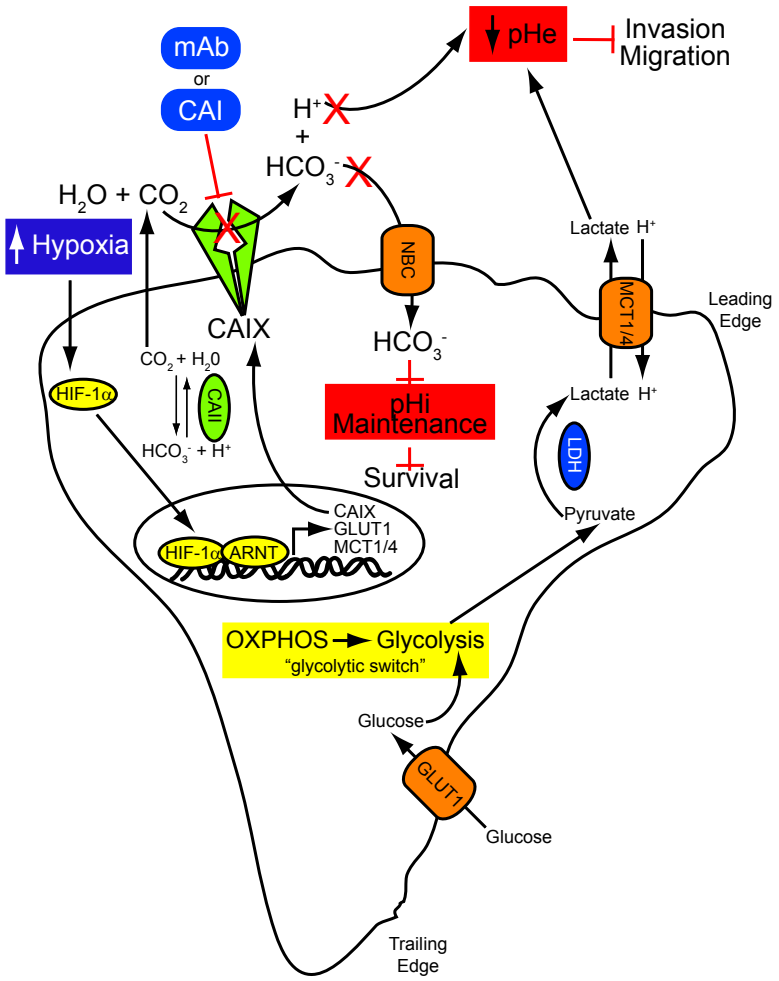


Figure 5. Molecular and metabolic adaptation of breast cancer cells upon HIF-1α mediated signalling. Signalling cascade results in nuclear translocation of HIF-1α and consequent activation of genes, such as GLUT1, MCT1 and MCT4, as well as CAIX. Then, cancer cells reprogram their metabolism towards the glycolytic pathway, resulting in an increased production of lactate and in high intracellular acidification. Overexpression of CAIX results in the production of $HCO3^-$, which is actively transported to the inside of cancer cells, compensating the intracellular acidification and maintaining cell survival. Activation of CAIX also results in acidification of the extracellular medium by the production of protons, promoting cancer cell invasion. Adapted from McDonald *et al.* [79]

CAIX, a major downstream target of HIF-1α, plays an important role in the pH regulation and adaptation to acidosis through the reversible hydration of CO_2 [83, 84]. The resultant $HCO3^-$ is transported inside the cell, in order to decrease the acidity created by increased metabolic activity; moreover, the protons derived from CAIX activity will promote ECM degradation, potentiating growth and invasion of surviving cells [85, 86]. *In vivo* studies, using orthotopic mouse models, have demonstrated that

CAIX depletion in breast cancer cells impairs primary tumour growth and also metastasis formation in nude mice [87]. CAIX is present in tumours with the ability to respond to hypoxia, including breast carcinomas, and is associated with poor patients prognosis, short disease free survival, basal-like phenotype; it is also described to be an independent poor prognosis factor [71, 88-91]. Moreover, this protein is known to induce tumour growth and cell migration, as well as cell invasion [92]. Inhibition of CAIX activity, using monoclonal antibodies or small molecule inhibitors, prevents cellular acidification and consequently decreases cell survival and invasion ability of cancer cells [79].

Other important cellular pH regulators are MCTs (monocarboxylate transporters), which are responsible for the extrusion of the excessive lactic acid resulting from increased glycolysis [93]. MCTs are found to be upregulated in several types of tumours, such as high-grade glial neoplasms, colorectal and cervical cancer [94-98]. In breast cancer, MCT1 is upregulated in poor prognosis basal-like breast carcinomas [99], being also associated with high histological grade tumours, negativity for hormone receptor and expression of CK5, CK14 and Vimentin [71]. Moreover, MCT1 and MCT4 expression are significantly associated with CD147 expression in breast carcinomas [99]. CD147, or EMMPRIN (extracellular matrix metalloproteinase inducer), is a cell surface glycoprotein which expression is required for MCT1 and MCT4 proper expression, location and activity [100, 101]. However, CD147 is also known to induce MMP production in cancer cells as well as in their neighbouring fibroblasts [102]. Sun *et al.* demonstrated that CD147 transfection in MDA-MB-435 induced an increase of expression of MMP, particularly of MMP2 [103]. In addition, it was also demonstrated, in *in vivo* experiments, that EMMPRIN induced expression in breast cancer cells resulted in the enhanced tumour growth in nude mice, associated with increased MMP expression [104]. Thus, it seems that CD147 or EMMPRIN-mediated MMP induction is a mechanism that is present in pathological conditions, such as breast cancer. In addition, Pinheiro *et al*. demonstrated that, in breast cancer, CD147 expression is associated with basal-like phenotype, high histological grade, hormone receptor negative status, basal cytokeratins 14/5 and also with Vimentin expression [99].

Moreover, the expression and activity of lactate dehidrogenase (LDH), an enzyme responsible for the interconversion between pyruvate and lactate, has also been shown to be essential in breast cancer metabolism. Specifically, LDH-A is upregulated in breast tumours [105-107] and downregulation of its expression and activity was associated with stimulation of mitochondrial respiration, decreased membrane potential, decreased ability of cells to proliferate under hypoxic conditions, as well as tumourigenicity in mice models [4]. Moreover, LDH-A expression silencing in metastatic breast cancer cells is associated to decreased invasion, migration and glycolysis, enhancing oxygen consumption, ROS and ATP content. These alterations were accompanied by decreased primary tumour growth and metastatic ability of these highly aggressive murine 4T1 breast cancer cells [106]. Acidosis, by lactate accumulation due to metabolic changes, associates with increased aggressiveness in breast cancer. In 2011,using metabolic measurements of LDH-A and lactate levels, Serganova *et al.* observed that aggressive 4T1 xenografts exhibit higher LDH-A expression and produced higher levels of lactate when compared to 67NR tumours, which show less aggressive and metastatic potential [107]. Moreover, the inhibition of this molecule inhibited the formation of metastasis and was accompanied by *in vivo* changes in tumour cell metabolism [106].

Interestingly, the acquisition of hypoxia, glycolysis and acid resistance phenotype was proposed to play important causal roles in breast carcinogenesis (**Figure 6**). Chen *et al.* provided evidences for a model of somatic evolution adaptation to microenvironmental conditions. These authors claim that hypoxia, glycolytic and acid-resistant phenotypes, represented by the expression of HIF-1α, GLUT1 and CAIX expression in tissue samples of normal mammary gland, ductal hyperplasia (DH), atypical ductal hyperplasia (ADH), *in situ* and invasive ductal breast carcinomas [89], symbolize a powerful adaptive advantage and an aggressive phenotype to breast tumours. Taken together, it is undeniable that this phenotype is highly associated to malignant features of breast cancer.

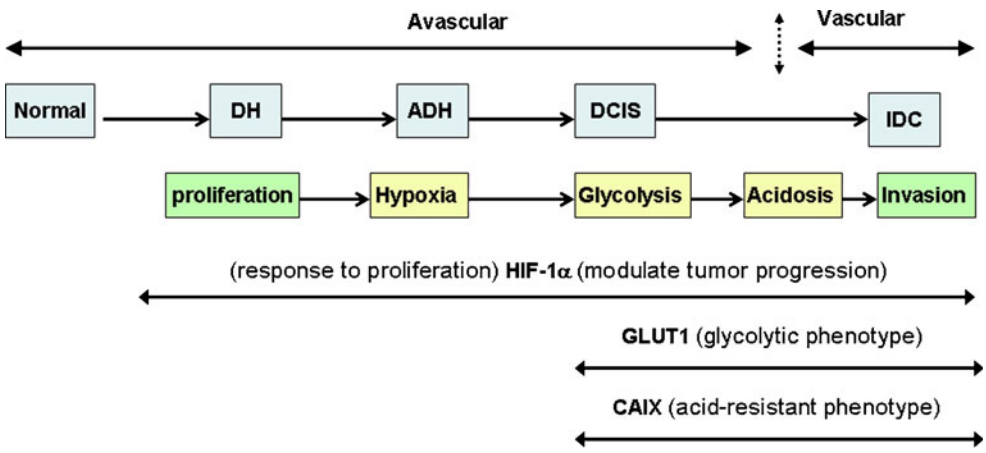


Figure 6. A model for somatic evolution during breast carcinogenesis. Ductal hyperplasia leads to a thickening of the epithelium, resulting in profound hypoxia. Hypoxia leads to upregulated glycolysis, which results in acidosis in the microenvironment. The role of HIF-1α changes from response to proliferation to tumour progression during breast carcinogenesis. Adapted form Chen *et al.* [89].

Interestingly, BLBC present a more emphasized response to hypoxia than tumours with luminal characteristics. It has been shown that this group of tumours present a differential expression of proteins whose genes are induced by hypoxia and are responsible for the development of a hypoxic and glycolytic/acid resistant phenotype [71, 91, 99], that confers selective advantage to tumours, allowing them to grow and escape cell death in adverse conditions. Several reports have been emerging in the literature, attributing a role to hypoxia-induced effects in BLBC aggressiveness. In 2011, Voss *et al.* showed that resistance to anti-angiogenic factors was linked to hypoxia-induced migration, mediated by an autocrine action of released signal substances in BLBC cells [108]. Moreover, several hypoxia-induced molecules have been reported as playing a role in aggressive behaviour of these breast tumours [109].

1.5 Metabolic Alterations in Breast Cancer

As stated before, metabolic reprogramming is currently considered as one of the major hallmarks of cancer [8]. In fact, several studies have been highlighting the importance of metabolism in breast cancer initiation and progression, trying to provide putative diagnostic and prognostic biomarkers, as well as new therapeutics targets

[89, 110-113].

Interestingly, Lu *et al.* proposed the “Two-step theory of breast cancer progression”, where metabolite alterations seems to play a role either in initial transformation of normal murine mammary gland cells as well as in the acquisition of metastatic ability of breast cancer cells[110]. Using high throughput metabolomics analysis in 4T1 syngeneic mouse model of breast cancer progression, these authors identified the deregulation of hundreds of metabolites involved in metabolic pathways and provided valuable knowledge implicating metabolites belonging to TCA, glycolysis nucleotides synthesis and antioxidant processes, in breast cancer initial transformation and progression [110]. Moreover, it was recently shown that breast cancer progression is accompanied by a decrease in cellular OXPHOS capacity, independently of mitochondrial copy number or electron transport chain (ETC) protein expression [112]. Using a model of breast cancer progression of immortalized mammary epithelial cell line (MCF10A), Shaw *et al.* demonstrated a decrease of cellular oxygen consumption rate with increase aggressiveness of human breast cancer cells [112]. Still, comprehensive metabolic profiles identified metabolite deregulation in invasive breast carcinomas compared with normal breast tissue, also implicating changes in metabolic pathways in breast cancer progression [113, 114]. In 2012, Budczies *et al.* analysed the metabolic changes in the central pathways between invasive carcinoma and normal breast tissues and identified key metabolic markers that separate cancer from normal tissues with high sensitivity and specificity [113].

Moreover, oestrogen stimulation induces several metabolic alterations that contribute to the metabolic cancer phenotype and allows breast cancer cell proliferation. Estradiol, the most active form of oestrogen, enhances glucose and glutamine consumption, as well increased lactate production [115, 116]. Additionally, estradiol increases PPP by upregulation of glucose-6-phosphate dehydrogenase, favouring biosynthetic processes for building blocks necessary for cell proliferation [116, 117].

Upregulation of glycolysis leads to microenvironmental acidosis requiring evolution to phenotypes resistant to acid-induced cell toxicity. Thus, cell population with increased glycolysis and acid resistance has a powerful growth advantage, promoting tumour progression. Thus, distinguishing unique metabolic adaptations of cancer has

obviously been seen as a valuable therapeutic target. Consequently, intensive research has been carried to pursuit an effective strategy to target those metabolic reactions.

2. Metabolism and Stemness

It has been widely accepted that metabolic pathways play a critical role in somatic reprogramming [118]. Moreover, distinct metabolic properties and mitochondrial functionalities have been attributed to different types of stem cells and to their more differentiated counterparts. Moreover, the microenvironment where stem cells are known to exist seems to be also important for the maintenance of their pluripotent state, as well as for their metabolic program [2].

2.1 Stem cell Microenvironment and Metabolism

2.1.1 Stem Cell Niche Microenvironment

Characterization of stem cell neighbours, expression patterns of signalling molecules and local environmental factors, such as extracellular matrices and oxygen pressure, suggests that stem cells reside in individual compartments, called stem cell niches, with specific microenvironmental conditions. However, the identification of these niches and the understanding of how they are regulated, is still a challenge. The stem cell niche is defined by the microenvironment surrounding stem cells, that not also maintains their stemness as well as it prevents them from differentiating [119].

Early mammalian development occurs in a relatively oxygen-poor environment, because before the establishment of the circulatory system, delivery of oxygen to the embryo is subject to the limits of diffusion (approximately 150 mm) [1, 120]. Thus, embryogenesis is heavily influenced by oxygen gradients. Evidences for this concept were found measuring oxygen tensions in endometrial and trophoblastic tissues during early pregnancy [121, 122]. In adult tissues, several stem cell niches have been reported as presenting low oxygen tensions, and due to the abnormal

vasculature, different hypoxia tensions are experienced, and can be as low as 1% of oxygen, which is relatively low comparing to other tissues (**Figure 7**). These oxygen tensions observed in animal tissues, specifically in stem cell niches, make the scientific community to link hypoxia signalling with stem cell behaviour [2, 123].

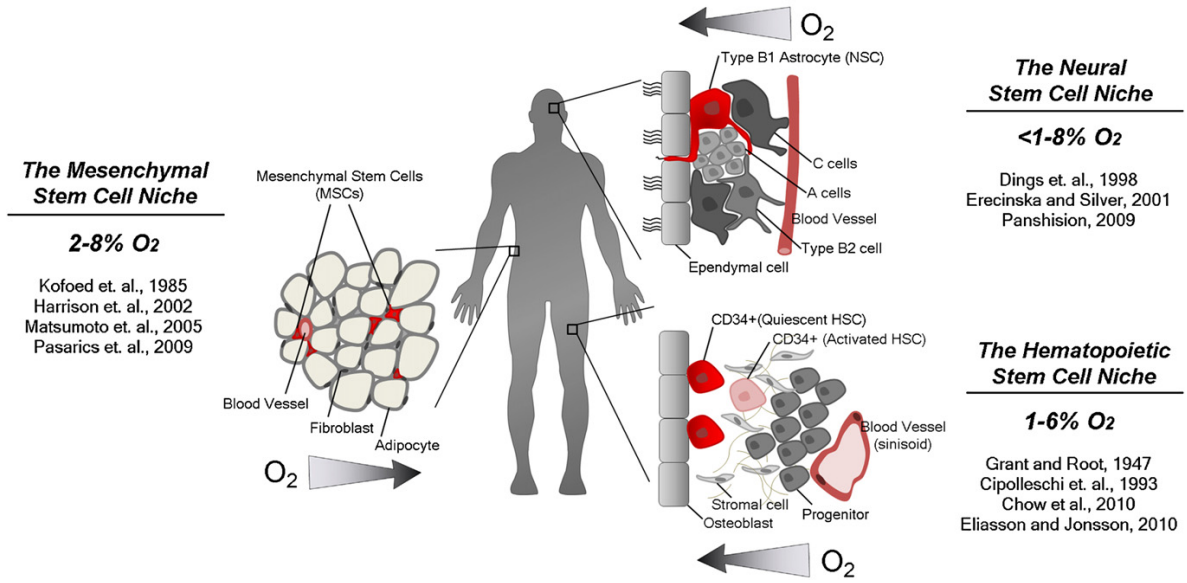


Figure 7. Low Oxygen Tension Measurements in Various Stem Cell Compartments. Representation of current available data for the hematopoietic (HSC), mesenchymal (MSC), and neural stem cells (NSC) in their designated niches: the bone marrow, adipose tissue, and the subventricular zone, respectively. Red cells represent HSCs, MSCs, and NSCs. Various oxygen tension measurements from the tissues and blood supply where hematopoietic and mesenchymal stem cells reside have been reported in the literature. Adapted from Mohyeldin *et al.* [2].

Low oxygen tensions in stem cell niches offers a selective advantage that is well suited to their particular biological roles [124]. Hypoxia has been shown to activate molecular pathways in multiple stem cell systems that appear to regulate Oct4 and Notch signalling, important regulators of stemness [125]. Moreover, proliferation and stem cell quiescence may also be regulated by oxygen tension of their niche. It is reported that oxygen tensions, as low as 1%, appear to decrease proliferation and maintain embryonic stem cell (ESC) pluripotency, while higher oxygen tensions (3% to 5%) seems to maintain pluripotency, with no effect on proliferation [126]. Interestingly, hypoxia also plays a role in the maintenance of self-renewal, undifferentiated state and pluripotency of ESC, HSC (hematopoietic stem cells), NSC

(neural stem cells), iPSC (induced-pluripotent stem cells), as well as CSC [124, 127-131]. For example, hypoxia seems to be important in the generation of iPSC, since HIF-2α and HIF-3α increases endogenous expression of transcription factors used to generate iPSC from differentiated somatic cells [2].

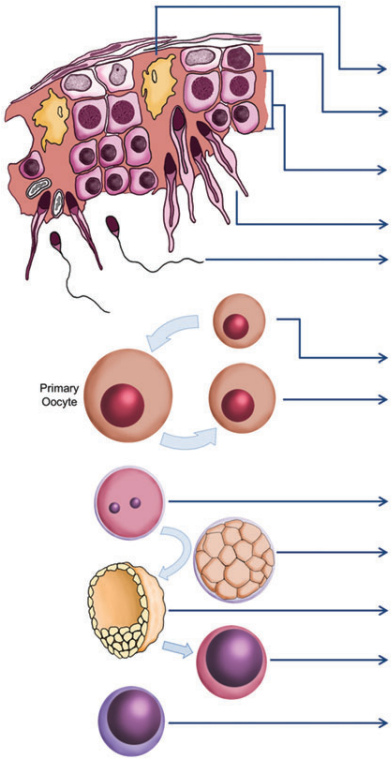
2.1.2 Stem Cell Metabolism

Different types of cells involved in spermatogenesis, oogenesis and embryogenesis, present specific mitochondrial characteristics, exhibiting peculiar metabolic phenotypes (**Figure 8**) [9].

Consistently, it is described that, during the early stages of embryonic development, there is a metabolic shift from oxidative phosphorylation (OXPHOS) to glycolysis, and oxidative metabolism is only fully reinstituted after implantation [132].

Mitochondrial and metabolic remodelling may underlie acquisition and maintenance of pluripotency. Pluripotent ESCs can differentiate into any cell type in the adult organism. They present specific mitochondrial morphology, with arranged in small perinuclear clusters, with immature morphology [133-135]. Differentiation involves alterations in mitochondrial networks, suggesting higher OXPHOS activity [133, 136]. Several reports have demonstrated that differentiation of ESCs *in vitro* induces changes in mitochondrial dynamics, namely in the increasing number and morphology of mitochondria [14, 133, 135-137]. Concomitantly, there is an increase of the O_2 consumption rates and ATP production as well a decrease in lactate production, suggesting that a switch in energy metabolism from glycolysis to oxidative phosphorylation is required for proper cell differentiation [138]. The increase in the number of mitochondria and OXPHOS in differentiated cells also leads to an increase in ROS production [133, 139], which has been proved to be important for the differentiation of hESCs into cardiomyocytes [140]. Several authors have reported similar characteristics in adult stem cell differentiation [141-143]. iPSC are somatic cells that have been reprogrammed to pluripotency, usually by the use of pluripotency-associated molecules such as Oct3/4, Sox2, Klf4 and c-Myc, with distinct methodologies [144]. Interestingly, iPSCs exhibit similar profile of mitochondrial

activity and metabolic behaviour as ESCs. These similarities comprise characteristics such as decreased mitochondrial mass, mtDNA copy number and expression of mitochondrial biogenesis genes as well as increased upregulation of glycolytic enzymes, and consequently a glycolytic metabolic programme [145-147].



Cell Type	Mitochondrial Morphology	Metabolic Phenotype
Spermatogenesis		
Sertoli Cells	Lamellar cristae connected by cristae junctions to the IMM, elongated mitochondria	Highly glycolytic
Spermatogonia	Ovoid shape with electron lucid (known as orthodox mitochondria)	Highly glycolytic
Spermatocytes	Transition from an orthodox mitochondria (less active) to a condensed form (more active): mitochondria are more elongated with cristae shaped as a crescent and matrix is less condensed	Transition from glycolysis to OXPHOS
Spermatids	Condensed	OXPHOS
Mature Sperm	Intermediate morphology and tightly wrapped around the axoneme	Glycolytic OXPHOS
Oogenesis		
Oogonium	Either spherical or ovoid shape with a pale matrix	Glycolytic
Mature oocyte	Increase in number and mitochondria maturation with denser matrix. Cristae pattern alters to a more energetic phenotype	OXPHOS
Embryogenesis		
Zygote	Oval mitochondria with few cristae and an electrodense matrix	OXPHOS
Morula	As the number of cells in the morula increase the mitochondrial morphology also alters to a more elongated morphology and transverse cristae	Glycolysis/OXPHOS
Blastocyst	Trophoblast cells present mature mitochondria with transverse cristae while the ICM cells present immature mitochondria with few cristae and less dense matrix	Mainly Glycolytic although OXPHOS might also occur
Embryonic Stem Cells	Round, immature phenotype, with a less dense matrix and little number of cristae	Mainly glycolytic
Induced Pluripotent Stem Cells	Intermediate phenotype between embryonic stem cells and differentiated cells. Round and elongated mitochondria with transverse cristae and a dense matrix	Mainly glycolytic

Figure 8. Mitochondrial characteristics and energy metabolism of different types of reproductive cells and embryos. Different mitochondrial morphologies and consequent metabolic pathways thought to be prevalent in the different stages of gametogenesis and early development. Adapted from Pereira *et al.* [9].

2.2 Breast Stem Cells

In the last decade, it has been firmly established that, like most tissues, if not all, the mammary gland has also a hierarchical organization, similar to the hematopoietic system [148]. At the top of this cell-hierarchy, there is a small population of cells with self-renewal capability, breast stem cells, responsible for generating and maintaining the tissue architecture and permitting tissue remodelling and repairing.

Independent of the developmental stage, mammary epithelium taken from any area of

the mammary gland is able to fully reconstitute a ductal tree when grafted to cleared fat pads, indicating that stem cells and their niches are distributed at regular intervals throughout the ductal system along development (**Figure 9**).

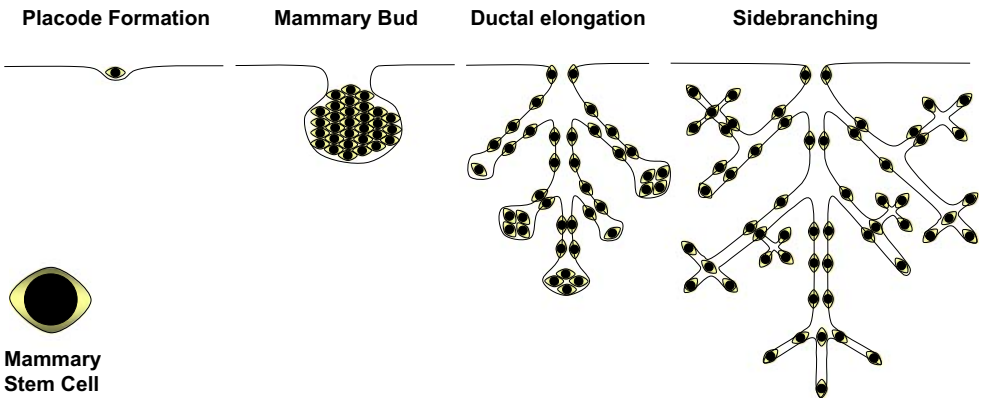


Figure 9. Stem cell distribution at different stages of mammary gland development. Schematic representation of distinct stages of mammary gland development with respective distribution of stem cells, not drawn to scale. Mammary epithelium taken from any area of the mammary gland is able to fully reconstitute a ductal tree when grafted to cleared fat pads indicating that stem cells and their niches are distributed at regular intervals throughout the mammary gland. Adapted from Brisken and Duss [149].

Stem cells in the mammary gland are scarce and, although they have been purified and isolated using different techniques, they have not yet been fully characterized. A number of different cell surface markers have been described to define stem and progenitor mammary cells from both human and mice origin. In 2001, using Fluorescence Activated Cell Sorting (FACS), Stingl *et al.* fractionated the human mammary epithelial gland using the markers EpCAM and CD49f and the isolated subpopulations were characterized for lineage restricted *in vitro* colony forming ability [150]. Since then, several groups have used different markers to isolate and characterize *in vitro* colony forming ability, as well as *in vivo* mammary repopulating capacity [150-154], identifying subsets of cells that were also enriched in mammary stem cells but expressing the different sets of markers[154-157].

Hormone signalling, such as ER signalling is important to stem cell function and mammary epithelium development [149] and human breast stem cells are likely to be ER negative and to require the presence of ER positive cells, the so-called sensored

cells [153, 157-159]. Moreover, it has also been shown that putative mammary stem cells in the mouse typically show the expression of EGFR (epidermal growth factor receptor) and high molecular weight cytokeratins, weak or almost absent expression of CK18 and lack the expression of ER and HER2 [158, 160]. Thus, it is hypothesized that mammary stem cells reside within the basal compartment of the mammary gland. However, no definitive phenotype is described for the stem cell at the top of the hierarchy or the progenitor cells.

2.3 Cancer Stem cells (CSC)

2.3.1 Cancer Stem Cell Definition and Characterization

Normal adult stem cells have self-renewal and differentiation capacity into several lineages; tumour cells have high proliferative capacity, phenotypic plasticity and aberrant differentiation [161]. These similarities between stem cells and tumour cells have given rise to the hypothesis that breast tumours arise from undifferentiated stem or progenitor cells. Moreover, stem cells and CSC share innumerable properties and characteristics, such as self-renewal and the reliance on similar signalling pathways (for example, Wnt or Notch pathways) and markers such as CD133 [161].

The CSC hypothesis or hierarchical model, postulates that a small subpopulation of cancer cells is tumourigenic and has the ability to self-renew and generate the heterogeneity of cells that comprise the tumour [161, 162]. These cells, named cancer stem cells (CSC) or tumour-initiating cells (TIC), share similar properties with normal tissue stem cells, including self-renewal and differentiation capacity.

One major breakthrough in cancer biology has been the isolation and characterization of CSC [163]. CSCs have now been isolated from several human tumours, including leukaemia [164], breast [165], brain [166], melanoma [167] and colon [168], and the presence of these tumour cell subsets correlate strongly with tumour recurrence and treatment failure.

Most authors use cell surface proteins, in order to define a subpopulation of cells that represents the breast CSC population. In 2003, Michael Clarke's group isolated a

subset of breast cancer cells with the phenotype $ESA^+/CD44^+/CD24^-$/low, which were able to self-renew and were highly tumourigenic at a low cell inoculum [165]. Since then, several other phenotypes and markers have been described to be able to identify and isolate breast CSCs. However, the complexity of CSC markers continues to pose challenges for identifying and isolating the putative tumour stem cell populations by the cell-sorting approach [169].

Immunohistochemically, basal-like breast tumours share a profile similar to the stem/progenitor cells of the normal female breast, including c-kit, α6-integrin, CK5, CK14 and prion protein. It is believed that the stem cell at the top of the hierarchy is triple negative ($ER^-/PgR^-/HER2^-$), $EGFR^+$, $p63^+$ and $p21^-$, similarly to myoepithelial cells of the mammary gland. One of the most accepted phenotype to identify breast CSC in poor prognosis basal-like breast tumour is the $CD44^+/CD24^-$ phenotype. Basal-like breast tumours are mainly composed of cells expressing the CSC marker CD44 [157, 170, 171] and an association between the $CD44^+/CD24^-$ phenotype and BLBC subtype has been reported [172-174], indicating possible stem cell features for this molecular subtype of cancer. However, the CSC phenotype $CD44^+CD24^{-/low}$ is not universal and other phenotypes have been proposed to better describe CSCs present in basal-like mammary tumours. Other cell markers associated with stem cell properties were identified in BLBC, such as the enzyme aldehyde dehydrogenase-1 (ALDH1), which was correlated with poor prognosis cancers [155, 173]. Also, in human ER-negative breast cancers, the phenotype $CD44^+CD49f^{high}CD133/2^{high}$ defines a population of tumour initiating cells with self-renewal capacity [175].

Moreover, human *BRCA1* gene inactivation, which is often associated with this phenotype of breast cancer, causes a defect in progenitor cell lineage commitment, with an increase in the immature CK14+/CK19+ cells [176], as well as an increase in ALDH1 activity with loss of mature luminal differentiation markers [177]. In mice breast tumours harbouring *BRCA1* deletion, additionally to the $CD44^+CD24^{-/low}$ subpopulation, the population defined as $CD133^+$ ($Prominin^+$) correlated with stem cell activity, producing increased spheroids, being more resistant to DNA damaging drugs, and having increased tumourigenic ability [178].

In addition to the ability to initiate tumours, CSCs are thought to be capable of

initiating metastasis, not only due to their increased ability to invade through Matrigel matrix [179], as well as by their link to EMT [156]. Furthermore, the prevalence of $CD44^{+}CD24^{-}$ cells in breast cancer patients indicates a link between high numbers of stem-like cancer cells and metastasis [180]. Recurrence of breast cancer may reflect the inherent ability of CSCs to survive as circulating tumour cells and to form micrometastasis, remaining quiescent in distant sites for a long period. The metastatic behaviour of cancer cells is also associated to the acquisition of a mesenchymal phenotype, which enables certain stem-like characteristics [156, 181].

From a clinical point of view, CSCs are a major concern due to their resistance to oncologic therapy regimens. Classical antineoplastic treatments, such as chemotherapy or radiation, can efficiently eradicate the majority of proliferating and genetically unstable malignant cells within neoplastic lesions. However, these treatments are inefficient in the eradication of the subpopulation of CSCs because these hold innate resistance mechanisms against radiation- and chemotherapy-induced cell death, allowing them to survive and cause tumour recurrence. The mechanisms of intrinsic therapy resistance in normal and malignant mammary stem cells involve enhanced DNA repair mechanisms and anti-apoptotic, which confer resistance of stem cells to DNA-damaging agents [182-185], as well as increased stem-cell maintenance signalling pathways, such as Wnt/β-ctn and Notch signalling [186, 187]. Still, CSCs express high levels of multidrug resistance transporter proteins [188].

2.3.2 Cancer Stem Cell Niches and Microenvironment Influence

Oxigen influences not only embryonic, but also adult stem cell and CSC biology [127]. CSC niches represent a subject of deep research due to the strong correlation with tumour recurrence and treatment failure in several types of cancer. Two different CSC niches with different localizations have been described (**Figure 10**). The first one, the perivascular niche, near the blood vessels, such as the one suggested for brain tumour initiating cells [189]. The existence of these niches is reinforced by the identification of integrin $\alpha 6$ [190] and nitric oxide (NO) [191] as regulators of glioma

stem cells (GSC). In fact, it was proposed that hypoxia inducible factors might be activated by NO in the perivascular niche of several known cell lines *in vitro*, despite the levels of oxygen in this region [192]. The second CSC niche is thought to be a hypoxic niche, away from vasculature [130]. The hypoxic environment observed in these niches seems to activate signalling pathways, through the activity of hypoxia inducible factors, which are responsible for known characteristics of CSC, such as development of an EMT phenotype, maintenance of stemness and self-renewal, as well as a metabolic shift. Thus, microenvironmental conditions of these niches have been proved to be fundamental for maintenance of stem and CSCs. Essentially, this condition has also become acceptable for the increased activity of several types of CSCs, such as human acute myeloid leukaemia (AML) and gliomas [130, 193]. In gliomas, HIF-2α, but not HIF-1α, has been shown to reduce activity of CSC in patients xenografts [130]. In contrast, HIF-1α was shown to be important in CSC activity in rodent transplant model of lymphoma and AML patient xenografts, through Notch signalling pathway [193].

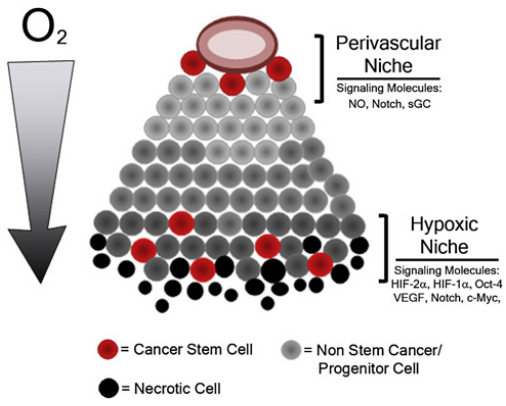


Figure 10. Cancer stem cell niches. CSCs were initially described to reside in a perivascular niche around tumour vasculature. Recent evidence suggests that NO produced by endothelial cells maintains the CSC phenotype. Inhibition of this signalling pathway results in loss of neurosphere forming capacity and attenuation of tumourigenic forming capacity in vivo. A secondary niche more distal from the vasculature exhibits lower oxygen tension and has also been shown to regulate the cancer stem cell phenotype. Adjacent to the rim of necrotic cells, this hypoxic niche contains cancer stem cells whose activity is modulated by multiple HIF-regulated genes, such as HIF-2α and HIF-1α, and other signalling molecules (Oct4, VEGF, Notch, and c-myc). Adated from Mohyeldin *et al.* [2].

In breast cancer, emerging data has established the link between hypoxia and CSC activity. Schwab *et al.* have demonstrated that HIF-1α promotes mammosphere

formation and enhances CSC frequency *in vivo*, in part by the regulation of the expression of markers associated with the basal lineage and Notch pathway [46]. Furthermore, it was shown that repetitive hypoxia/reoxygenation cycles are able to select and expand a highly tumourigenic, with EMT phenotype, stem-like breast cancer cell subpopulation [39]. Still, Gammon *et al.* demonstrated that hypoxia selects a breast cancer cells population with EMT features and increased mammosphere ability, a typical characteristic of CSCs [38]. Interestingly, differential expression of HIF-1α was observed in human primary invasive breast carcinomas with $CD44^{+}CD24^{-/low}$ phenotype [194].

Importantly, breast CSC expansion by tumour hypoxia seems to play a causal role in the limited efficacy of anti-angiogenic therapies. Blocking angiogenesis through inhibition of the vascular endothelial growth factor (VEGF) pathway reduces tumour growth. Clinically, VEGF-neutralizing antibody Avastin (bevacizumab) and VEGF receptor tyrosine kinase inhibitors (sorafenib and sunitinib) have been used as anti-cancer treatments in several tumour types, including breast cancer [195]. However, only a moderate increase in progression-free survival and little benefit in overall survival have been observed [196]. This inefficacy was partially attributed to the generation of intratumoural hypoxia, which leads to an increase of breast CSC population, through Wnt pathway via Akt/β-ctn signalling [197].

Moreover, hypoxia-induced CAIX is also involved in the expansion and maintenance of CSC. In 2012, Lock *et al.* described CAIX as a critical mediator of the expansion of breast CSCs in hypoxic niches by supporting the mesenchymal and 'stemness' phenotypes of these cells, pointing to CAIX as an important therapeutic target for selectively depleting breast CSCs and improvement of chemotherapeutic response [198]. Glucose restriction is also involved in CSC expansion. This is the case of glioblastomas, for example, where nutrient limitation contributes to tumour progression by the enrichment of brain CSC. Flavahan *et al.* demonstrated that non-brain CSC are able to adapt and survive to glucose deprivation environments by the acquisition on brain CSC features. Importantly, these authors demonstrated that growth and tumourigenic potential of these aggressive tumours is highly influenced by GLUT3 mediated glucose uptake by brain CSC [199]. Thus, suppressing the hypoxic

response and/or targeting pathways of nutrient uptake and metabolism, may be beneficial for cancer patients due to their involvement in breast CSC subpopulation activity and expansion, that are thought to be responsible for patient relapse and metastasis.

2.3.3 Breast Cancer Stem Cell Metabolism

Interestingly, stem and CSCs also share unique metabolic properties, in common with actively proliferating cells. The increased glycolytic profile, low OXPHOS and low mitochondrial activity, characteristics observed in pluripotent stem cells, are shared with CSC. Recently, Vega-Naredo *et al.* showed that embryonal carcinoma stem cells present a glycolytic phenotype, decreased mitochondrial activity and biogenesis and increased resistance to DCA (dichloroacetate), comparing to their differentiated counterparts [200]. Specifically in breast cancer, cells with stem-like properties present a glycolytic metabolism comparing to their differentiated counterparts. Feng *et al.* have recently demonstrated that breast CSC, defined by $CD49f^{high}/Epcam^{low}$ phenotype, use preferentially glycolysis instead of OXPHOS as their metabolic programme, when compared with non-breast CSC [201]. Expression and activation of mitochondrial pyruvate dehydrogenase (PDH), which makes the link between glycolytic pathway and OXPHOS, favouring OXPHOS, was also found to be decreased in breast CSC when compared to non-breast CSC, explaining the unique metabolic programme of these cells [201]. Other authors have also demonstrated the glycolytic behaviour of breast CSC. Using proteomic and targeted metabolomics analysis, Ciavardelli *et al.* demonstrated that breast CSC shift from mitochondrial oxidative phosphorylation (OXPHOS) towards glycolytic metabolism and present increased expression of key enzymes of anaerobic metabolism, such as PKM2 (Pyruvate dehydrogenase kinase M2), LDH, and G6PD (glucose-6-phosphate dehydrogenase), as well as increased antioxidant defence system [202]. Still, Gammon *et al.* also demonstrated that a breast CSC population with EMT characteristics, presented high levels of HIF-1α, decreased mitochondrial mass and membrane potential, consumed less oxygen and presented lower levels of ROS [38].

Moreover, several studies have been reporting that glycolytic inhibitors and OXPHOS enhancers, selectively targets breast cancer cells [203], as well as breast CSC, highlighting the dependence of these cells on glycolytic metabolism [201, 202].

2.3.4 Oxidative Stress in Stem and Cancer Stem Cells

ROS are a highly reactive group of oxygen-containing molecules, including free radicals and peroxides, such as superoxide anion ($O2^{\bullet -}$) and hydrogen peroxide (H_2O_2), generated as common by-products of oxidative metabolism, or in response to the activation of several oxidative enzyme complexes [204-207]. At physiologic concentrations, ROS functions as signalling molecules, being involved in redox-dependent regulation of multiple signal transduction pathways, meeting different biological processes such cell adhesion, migration, proliferation, differentiation, and survival [204-207]. An imbalance between the production of ROS and cellular antioxidant mechanisms, that detoxify these reactive molecules, leads to an excessive accumulation of ROS, exerting damaging effects through oxidative stress in cells. The maintenance of highly regulated mechanisms to control ROS levels and functional specificity is therefore essential for normal cellular homeostasis and proper response to environmental stimuli.

Cells that undergo aerobic metabolism are subject to some degree of oxidative stress through the generation of ROS that can damage DNA. For instance, mouse embryonic fibroblasts accumulate more mutations and senesce faster when cultured under 20% O_2 than when cultured under 3% O_2 [208]. By residing in low oxygen tensions compartments, stem and CSC not only maintain slow cycling proliferation, quiescent and undifferentiated state, but they can also escape the oxidative stress damage associated with oxygenated tissues [124, 127, 208, 209]. As described above, hypoxia induces a metabolic switch that shunts glucose metabolites to glycolysis to maintain ATP production and prevent increase of ROS levels to a toxic level [55]. Thus, HIF-1α is likely to be a candidate to regulate this mechanism, not only due to its function as a glycolytic metabolism inducer, as well as due to its role in mediating cell-cycle arrest [210]. Several types of cells, including HSC and early

progenitors, contain low levels of ROS comparing to their more mature progeny [211, 212]. This difference seems to be critical for maintaining stem cell function. In the mammary gland, the two specific types of cells, the luminal and the basal/myoepithelial cell layer, were found to present different levels of ROS, which was attributed to differences in their mitochondrial content [213, 214]. Interestingly, normal human basal mammary epithelial cells present low levels of ROS, which are thought to be maintained by glutathione-dependent mechanisms, while the matching purified luminal progenitor cells contained higher levels of ROS, multiple-independent antioxidants and oxidative nucleotide damaging control proteins, consuming oxygen at higher levels [214]. Moreover, luminal progenitor cells are known to be more resistant to glutathione depletion than basal cells and also to be more resistant to oxidative stress caused by H_2O_2 and ionizing radiation [214]. Moreover, it was also demonstrated that mammary epithelial cells coordinate their responses to detachment through the increase of antioxidant systems, such as SOD2 (superoxide dismutase 2), decreasing ROS production from mitochondrial oxidation, and allowing them to escape ECM-detachment cell death [215]. Similarly, normal mammary epithelial stem cells, as well as subsets of CSCs in some human and murine breast tumours, contain lower ROS levels than their corresponding mature progeny cells and non-tumourigenic cells (NTCs), respectively [213]. Since ROS are critical mediators of ionizing radiation-induced cell death [216], the idea that CSC are able to survive to radiation induced cell death due to their low content of ROS is extremely attractive. Similar to normal tissue stem cells, subsets of CSCs in breast tumours contain lower ROS levels and enhanced ROS defences compared to their non-tumourigenic progeny, which may contribute to tumour radioresistance. Dihen *et al.* demonstrated that breast CSC develop less DNA damage and are preferentially spared after irradiation compared to NTCs [213]. Moreover, other reports have corroborated this concept through the demonstration that breast CSCs are a radioresistant subpopulation of breast cancer cells, which are expanded in number after short courses of fractionated irradiation [217].

Thus, metabolic properties of stem and CSC, as well the environment of the place were they reside, are implicated in the resistance to therapy by the opportunity to

escape to oxidative stress. Taken together, since CSC might be responsible for recurrence, metastasis and therapeutic resistance, combination of CSC target therapies, with current available therapeutic regimens, is a goal for the improvement of cancer treatment (Ablett *et al.,* 2012).

3. P-CADHERIN IN STEMNESS AND IN CANCER

P-cadherin (or placental cadherin) is a cell-cell adhesion molecule, whose expression is highly associated with undifferentiated cells in normal adult epithelial tissues, as well as with poorly differentiated carcinomas. Its expression has been already reported in human ESC and it is presumed to be a marker of stem or progenitor cells of some epithelial tissues. In normal breast, P-cadherin has an essential role during ductal mammary branching, being expressed by the monolayer of epithelial cap cells at the end buds. In mature mammary tissue, its expression is restricted to the myoepithelium. In breast cancer, P-cadherin is frequently overexpressed in high-grade tumours, being a well-established indicator of poor patient prognosis. Moreover, P-cadherin is an important inducer of breast cancer cell migration and invasion. Due to P-cadherin's role in cancer cell invasion and metastasis formation, a humanized monoclonal antibody, which antagonizes P-cadherin-associated signalling pathways, was already produced and used in clinical trials.

3.1 PROTEIN STRUCTURE AND FUNCTION

P-cadherin was the third classical cadherin to be identified and characterized in the mouse visceral endoderm cell line PSA5-E [218, 219]. Its expression was originally observed in mouse placenta throughout pregnancy [218, 220] and, although the name might suggest, P-cadherin is not expressed in human placenta [220].

The gene encoding P-cadherin (*CDH3*), sharing 66% of homology with the far more well characterized *CDH1* (the gene that encodes E-cadherin), maps to chromosome 16q22.1, a region that contains a cluster of several cadherin genes, just 32 kilobases upstream of the gene encoding human E-cadherin [221, 222]. It is composed by 16

exons and exhibits a high degree of conservation in intron positions, as well as a large intron after exon 2 [223]. Additionally, this gene harbours a 5′-located CpG island on its promoter region (**Figure 11**) [224, 225].

Mutations in the *CDH3* gene have been reported to be responsible for congenital hypotrichosis associated with juvenile macular dystrophy, which is a rare autosomal-recessive disorder characterized by abnormal growth of scalp hair, followed by progressive macular retinal degeneration that leads to early blindness [226].

The mature P-cadherin glycoprotein has a molecular weight of 118 kDa, and its structure is similar to that of classical cadherins [218]. It comprises three distinct domains (extracellular, transmembrane and cytoplasmic) and it mainly, but not exclusively, promotes homotypic interactions (between P-cadherins molecules) [218, 227]. The amino- terminal domain is essential for the creation of lateral dimmers that act together in a zipper-like structure between neighbouring cells (**Figure 11**) [228, 229].

The function and strength of P-cadherin mediated adhesion depends on its dynamic association with a group of cytoplasmic molecules, called catenins. These molecules serve to link the cadherin cytoplasmic tail to the actin cytoskeleton and facilitate clustering into the junctional structure, forming cadherin-catenin complexes (**Figure 11**) [229, 231]. This tail comprises two main domains: the catenin binding domain (CBD), which is known to be essential for cadherin function, and the juxtamembrane domain (JMD), which has been suggested to play a critical role in allowing cells to relocate [227]. The α, β, γ and p120 catenins are the documented interaction partners [232]. β-ctn (and also γ-ctn) is a signalling molecule that is involved in tissue patterning, and it is regulated by the CBD, tyrosine phosphorylation and transcriptional factors [233].

p120-ctn interacts directly with the JMD and is also regulated by tyrosine kinases, modulating cadherin intracellular trafficking, stability, adhesive capacity and cell motility [234-236]. The α-ctn links the cadherin-catenin junctional complex to the actin cytoskeleton [233].

In 1989, Shimoyama and co-workers [220] showed that NIH3T3 cells transfected with human P-cadherin cDNA expressed the functional cadherin molecule, which was able to mediate cell-cell adhesion. The adherens-type junctions play an important role in cell sorting during embryogenesis and in the maintenance of specific organ and adult tissue architecture [237, 238]. These are also essential in intracellular signalling mechanisms [233, 239], which regulate cell polarity, differentiation, growth and migration [240].

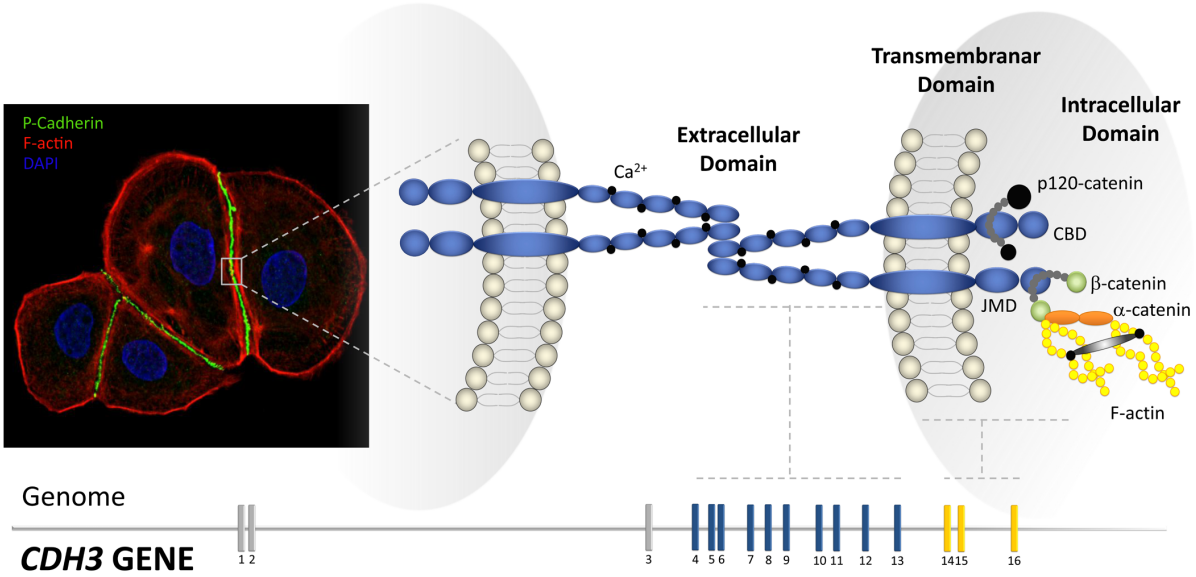


Figure 11. Schematic representation of the structural components of the P-cadherin adhesive junction. Lateral clustering of P-cadherin molecules is required to form stable cell-to-cell contacts in BT20 breast cancer cells [immunofluorescence: P-cadherin (green), F-actin (red), DAPI (blue)]. In the intercellular space, P-cadherin extracellular domains interact with P-cadherin extracellular domains of adjacent cells to mediate cell adhesion. The intra-cellular catenins bind to the cytoplasmic tail of P-cadherin. p120-ctn binds the cadherin tail at the juxtamembrane domain (JMD), whereas β-ctn binds to the distal catenin binding domain (CBD). α-ctn associates with β-ctn and is directly linked to the actin cytoskeleton. The lower panel shows the genomic structure of *CDH3*/P-cadherin gene, which is constituted by 16 exons: the extracellular part of P-cadherin is encoded by 10 exons (exons 4-13), whereas the transmembrane and intracellular domains are determined only by the information included in the last 3 exons (exons 14-16). Adapted From Albergaria *et al.* [230].

3.2 *CDH3* REGULATION

Regulation of cadherin-mediated adhesion seems to be a very dynamic, elegant and complex net of mechanisms and players, which underlie the dynamics of the

adhesive interaction between cells. Although the described evidences that the expression of inappropriate cadherins can result from growth factors and hormones stimulation in the tumour microenvironment, or from changes in the promoter regions of cadherins and transcriptional, and post-translational regulation, specific data concerning *CDH3* gene regulation is still very limited.

The pivotal molecular mechanism involved in CDH3/P-cadherin deregulation is mainly occurring at the promoter region of the gene and not by structural alterations of its coding sequences. The main described regulators of *CDH3* are ERα [241] and BRCA1/c-Myc/Sp1 [242], which act as repressors of P-cadherin expression, and β-ctn [243], C/EBPβ [244] and p63 [245], that are able to activate its expression (**Figure 12**).

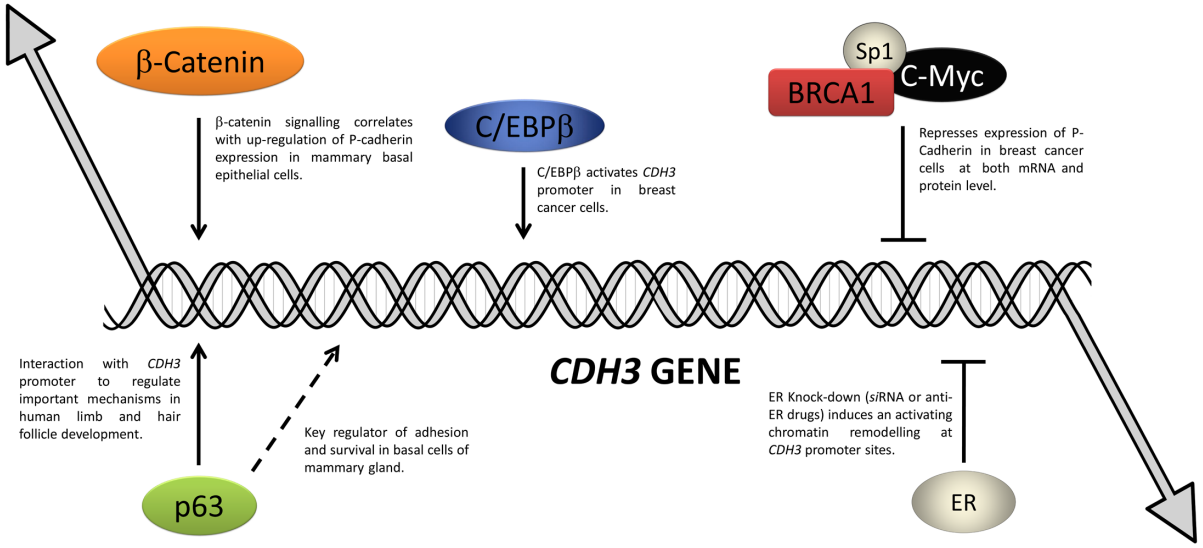


Figure 12. Transcriptional regulators of *CDH3/P-cadherin* gene promoter. β-ctn, p63 and C/EBPβ are transcriptional activators of the *CDH3* promoter. In contrast, BRCA-1/c-Myc/Sp1 complex acts as transcriptional repressor of the *CDH3* promoter. Moreover, oestrogen receptor (ER) can indirectly repress P-cadherin expression by controlling epigenetic changes in *CDH3* gene promoter. Adapted from Albergaria *et al.* [230].

In 2004, we described that the lack of ERα signalling is responsible for the P-cadherin *de novo* expression, categorizing *CDH3* as a putative oestrogen-repressed gene. It was also showed that the anti-oestrogen ICI 182,780 is able to increase *CDH3* promoter activity, mRNA and protein levels in a time and dose dependent manner [241]. Furthermore, this anti-ER drug induces a chromatin structural remodelling,

eventually allowing the binding of nearby transcriptions factors [246].

The expression profiling of BRCA1-deficient hereditary tumours has identified a pattern of gene expression similar to basal-like breast tumours [52]. Thus, as a gene associated with the basal-like phenotype in breast cancer, the mRNA and protein levels of *CDH3*/P-cadherin gene were shown to be also transcriptionally repressed by functional BRCA1 protein in breast cancer cell lines. This repression is achieved after the formation of BRCA1 and c-Myc repressor complex (BRCA1-c-Myc complex) on the promoters of specific basal genes, including *CDH3* gene, and represent a potential mechanism to explain the observed overexpression of key basal markers in BRCA1-deficient tumours [242]. In fact, in breast carcinomas, it has been shown that P-cadherin expression is strongly associated with the presence of BRCA1 mutations [247], which means that this repression does not occur in breast cancer cells with a BRCA1 mutation and the *CDH3* gene is expressed.

Conversely, *in vitro* and *in vivo* studies also have shown that β-catenin activates *CDH3* promoter, leading to overexpression of P-cadherin in basal mammary epithelial cells. In fact, it was shown that activation of β-catenin signalling correlates with upregulation of *CDH3* promoter and P-cadherin expression, as well as downregulation of endogenous β-catenin levels inhibited *CDH3* promoter activity [243].

Another putative transcription factor of P-cadherin is CCAAT/enhancer-binding protein β (C/EBPβ), which was demonstrated as able to upregulate *CDH3* promoter in breast cancer cells. Moreover, the expression of P-cadherin and C/EBPβ are highly associated in human breast carcinomas and both linked with a worse prognosis of breast cancer patients [246].

From the p53 family related factor,p63 is a key regulator of adhesion and survival in basal cells of the mammary gland, showing that several cell adhesion-associated genes were downregulated due to p63 expression, which also led to detachment between mammary epithelial cells [248]. This involvement of p63 in cell adhesion mechanisms was linked with *CDH3* gene in developmental models, where P-cadherin has been described as a direct p63 transcriptional target, interplaying a crucial role in human limb bud and hair follicle development [245]. Moreover, unpublished work form

our group demonstrates that different p63 isoforms have different abilities to modulate the activity of *CDH3* promoter in breast cancer cells, being the truncated TAp63γ isoform the one which greater represses *CDH3* activity. Additionally, we also observed a decreased of the P-cadherin induced functional proprieties, such as invasion and mammosphere formation efficiency (MFE), when cells were transfected with TAp63γ, being this effect dependent on p53 wild type or mutated status.

Moreover, epigenetic alterations are also responsible for regulation of *CDH3* expression. P-cadherin negative epithelial/luminal normal breast cells are consistently methylated in a specific region of the *CDH3* gene promoter, whereas in breast cancer, it was established the existence of a significant correlation between P-cadherin overexpression and hypomethylation of this same specific promoter region [249]. Regulatory role of DNA methylation in the expression of P-cadherin is also present in other cancer models. Analysis of *CDH3* promoter revealed that it was hypomethylated in colorectal and gastric cancer, leading to increased P-cadherin expression; however, in pancreatic and melanoma cancer models, *CDH3* was shown to be silenced by aberrant hypermethylation [250-252].

In 2005, we analysed P-cadherin promoter methylation in normal breast tissue, from which only epithelial cells were microdissected, and methylation of *CDH3* gene promoter was found in the normal epithelial/luminal cell layer from all the specimens analysed, which was associated with negative P-cadherin expression in these cells. Moreover, P-cadherin overexpression results from *CDH3* promoter hypomethylation. Using a large series of invasive breast carcinomas, we found that 71% of P-cadherin-negative breast cancer cases were methylated for the *CDH3* gene, whereas 65% of P-cadherin-positive cases were unmethylated [249].

However, promoter methylation is not the only epigenetic event that may regulate the *CDH3* gene promoter. Genomic structure of the gene, like the enrichment in CpG site, as well as the attributed DNA hypersensitive sites can also play a role in the regulation of *CDH3* expression. We have observed an upregulation of *CDH3* promoter activity and P-cadherin expression in cells treated with the histone deacetylases (HDAC) inhibitor Trichostatin A (TSA), showing that chromatin-activating modifications play an important role in the modulation of this gene [246].

3.3 Role in Stemness and Epithelial Cell Differentiation

Classical cadherins play important roles in maintaining the structural integrity of epithelial tissues and are mainly involved in cell differentiation during embryogenesis [230]. There are clear indications in the literature establishing the link between cell adhesion molecules and stem cell features, not only as biomarkers that help to isolate and characterise stem cells, but also as important mediators of stem cell activity, via modulation of signalling pathways [253].

P-cadherin was already identified as a marker of undifferentiated stem or progenitor cells [253, 254]. In a recent study, *CDH3* is one of the genes that encode a surface protein that identifies the pluripotent population of human ESC [255]. However, the most dramatic expression of P-cadherin is observed in the placenta, both in the embryonic and maternal regions, hence the classical denomination of placental-cadherin. Early reports specified low P-cadherin expression in human placenta, but high amounts in mice placenta [220].

In adult tissues, the expression of P-cadherin is mainly found in the basal layer of several epithelial structures, such as skin, uterine cervix, prostate, and lung, contributing to the maintenance of the epithelial phenotype. The expression of cadherin molecules was extensively studied in mouse epidermis, in adulthood and during fetal development, where it has been found that E-cadherin is expressed both in the basal and intermediate layers of epidermis, whereas P-cadherin is only expressed in the basal and proliferative layer [256]. Furthermore, loss of E-cadherin plays an important role in bud formation and in the acquisition of an invasive behaviour, whereas P-cadherin becomes predominant expressed later in development, namely in the growing hair follicle and in the early progenitor cells from hair germs and small hair placodes [257, 258]. Like hair follicles, sweat glands and mammary glands develop also from the same discrete accumulation of stem cells resting in the primitive epidermis, the outermost cell layer of an embryo, and there is strong evidence that dynamic changes in the composition of adherens junctions are important for the development of skin appendages [257].

The final evidence showing the importance of P-cadherin for the architecture and development of epithelial tissues was demonstrated by human genetic syndromes

that are induced due to P-cadherin loss. *CDH3* gene mutations have been shown to cause P-cadherin functional inactivation, leading to developmental defects associated with two inherited diseases in humans: 1) hypotrichosis with juvenile macular dystrophy (HJMD) and 2) ectodermal dysplasia, ectrodactyly, and macular dystrophy (EEM syndrome). No defects were described for these conditions, concerning the human mammary development [230].

P-cadherin is transiently expressed in various tissues during development [259, 260] and its permanent expression is limited to adult epithelial tissues, at cell-cell boundaries [261]. It has been associated with undifferentiation and proliferation of tissues, as well as with the connection or segregation of cell layers, as found for other cadherins [218, 257, 261, 262].

3.3.1 P-CADHERIN IN MAMMARY GLAND DIFFERENTIATION

During normal breast development, P-cadherin has an essential role in the ductal mammary branching, being expressed by the monolayer of epithelial cap cells at the terminal end buds (TEBs) [263]. Moreover, this molecule is important for the undifferentiated state of the normal mammary gland [264], where its expression is restricted to the myoepithelium, although it has been postulated that it may also be present in early luminal progenitor cells [228, 230, 265].

P-cadherin is extremely important to the establishment of the correct architecture of the tissue, as demonstrated by functional-blocking antibody experiments *in vitro* and *in vivo.* Daniel and collaborators exposed the end buds and mature mammary glands of 5 week-old virgin mice to slow-release plastic implants liberating a monoclonal antibody for P-cadherin. No effect in the luminal layer was found, but disruption of the basally located cap cell layer was clearly observed [263]. Also, more recently, Chanson *et al.,* described that P-cadherin contributes specifically to the organization of the myoepithelial cell layer of the breast, since when an antibody that blocks P-cadherin function was used in an *in vitro* self-organizing assay of the human mammary bilayer, the migration of mammary epithelial cells (MEC), occurring during normal sorting of both layers, was compromised [266]. These experiments indicate

that selective expression of P-cadherin in the basal layer is necessary for the maintenance of mammary tissue integrity.

In fact, deletion of P-cadherin affects normal mammopoiesis, since the *CDH3-null* female mice exhibit precocious mammary gland differentiation in the virgin state, and breast hyperplasia and dysplasia with age [264]. These observations in knockout animals indicate P-cadherin cell-cell interactions and signalling as regulatory determinants of the negative growth of the luminal epithelium, being important for the maintenance of an undifferentiated state of the normal mammary gland.

The expression of P-cadherin is activated in human mammary luminal cells during late pregnancy and lactation [267]. In these alveolar lactating cells, the pattern of P-cadherin expression is not restricted to the cell-cell borders, but shows a cytoplasmic staining, typical of a secreted protein. Indeed, in human milk, a soluble fragment of P-cadherin (sP-cad) with 80KDa was found to be present, corresponding to the extracellular domain of the molecule [267]. Mannello and collaborators showed that the highest concentration of sP-cad is detected in milk collected during the first trimester of lactation [268]. However, the biological and physiological role of this fragment in the normal function of the breast is still unclear. Some authors suggest a role for sP-cad in alveolar differentiation during lactation, or in the immune response of the mother or the baby, or as a signalling protein between epithelial and myoepithelial cells. This fragment was also found in different body fluids serum, like semen, nipple aspirate fluid (NAF) and breast cyst and amniotic fluid [267, 268].

3.4 P-CADHERIN IN BREAST CANCER

Because of their importance in normal development, disorders involving dysfunction of classical cadherins/catenins are related to various disease states, including cancer [232, 269, 270]. Particularly in breast cancer, P-cadherin has been recognized as a poor prognosis marker, due to its pro-invasive role in breast cancer cells, being considered as a putative therapeutic target in this disease.

3.4.1 Poor Prognosis Marker

P-cadherin expression has been found in several solid tumours, including breast, prostate, colon, pancreatic and bladder cancer [271], being considered as a tumour-associated antigen. In breast cancer, P-cadherin is aberrantly expressed in 25% of ductal carcinomas *in situ* (DCIS), as well as in 20% to 40% of invasive breast carcinomas [228, 272-274].

Importantly, several studies have reported P-cadherin as a marker of poor prognosis in breast cancer, since P-cadherin expressing tumours were significantly associated with short-term overall and disease-free survival, as well as with distant and loco-regional relapse-free interval [249, 275-277]. Accordingly, P-cadherin expression has also been positively associated with poorly differentiated and high histological grade tumours, as well as with well established markers of poor prognosis, like Ki-67, EGFR, CK4 and CK14 and negatively associated with age at diagnosis, hormonal receptors (ER and PgR), and Bcl-2 expression [38, 249, 271, 276, 277]. Besides the strong association between P-cadherin and poor prognosis, there is no significant association between P-cadherin expression and the histological subtypes of breast cancer. The majority of positive P-cadherin tumours are invasive ductal carcinomas NOS, or carcinomas with metaplastic or medullary features [249, 277, 278]. However, metaplastic and medullary breast carcinomas are consistently immunoreactive for P-cadherin supporting the hypothesis of a myoepithelial/basal transcriptomic programme for these lesions [279, 280]. P-cadherin is also overexpressed in an aggressive form of locally advanced breast cancer with high metastatic potential and high death rate, the inflammatory breast carcinomas (IBC) [281]. Moreover, Mannello *et al.* demonstrated a significant increase of soluble P-cadherin (sP-cad) in NAF in woman with breast cancer when compared with healthy subjects or with women with pre-cancer conditions, suggesting its possible release via proteolytic processing in cancer cells [268]. However, the levels of the soluble P-cadherin fragment were not correlated with the presence of P-cadherin positive breast cancer [282].

3.4.2 Biomarker of Basal-like Breast Carcinomas

P-cadherin aberrant expression is found predominantly in poor prognosis basal-like and HER2-overexpressing breast carcinomas [228, 247, 273, 274, 277, 283].
BLBC are characterized by a triple negative (TN) phenotype, lacking the expression of hormone receptors (ER and PgR) and HER2. BLBC express genes characteristic of basal epithelial cells such as high-molecular weight basal cytokeratins (CK5/6, CK14, CK17), vimentin, caveolins 1/2 and EGFR [247]. Histologically, BLBC are poorly differentiated carcinomas, present high nuclear and histological grade and frequently show medullary and metaplastic features [284-288]. A distinct pattern of metastasis to brain and lungs, known to be associated with poor prognosis, and less significant involvement of axillary lymph nodes, has also been described in BLBC [170, 288, 289]. Until now, the most suitable immunohistochemical criterion to identify BLBC is the triple negative phenotype along with positivity for CK5 and/or EGFR [290]. However, we have previously demonstrated that P-cadherin expression shows higher sensitivity to distinguish the basal phenotype of breast carcinomas, being a reliable additional marker to be used in the daily practice of breast pathology laboratories for the identification of BLBC [283].

3.4.3 Promoter of Oncogenic Effects

Although the effect of P-cadherin expression in cancer is unquestionable, its role in the carcinogenesis process remains object of debate, since it can behave differently depending on the tumour cell model and context. In melanoma, P-cadherin seems to have a tumour suppressive function [291]. However, in breast cancer and in other tumour models, such as bladder and pancreatic cancer, P-cadherin is described to exhibit tumour-promoting effects [230, 292-294].
We have found that overexpression of P-cadherin promotes breast cancer cell motility, migration and invasion abilities when cultured in matrigel matrix [295]. Although the mechanisms by which P-cadherin promotes oncogenic-associated effects, we described that it induces the secretion of matrix metalloproteinases, such

as MMP1 and MMP2, leading to the cleavage of the extracellular domain of P-cadherin (sP-cad), which is responsible for the induction of invasion of non-invasive breast cancer cells [295].

Furthermore, we have recently demonstrated that P-cadherin functional role is dependent of E-cadherin expression in breast cancer cells [296]. We showed that E- and P-cadherin co-expressing breast cancer cells significantly enhanced *in vivo* tumour growth and that the expression of both cadherins is significantly correlated with high-grade breast carcinomas, biologically aggressive, and with poor patient survival, being a strong prognostic factor in this disease. Moreover, we showed that P-cadherin, in this context, was able to promote these effects by the interaction with E-cadherin, and disrupting its interaction with both p120-ctn and β-ctn, perturbing normal adhesion complex. In the absence of E-cadherin expression, in the same cancer model, P-cadherin is able to suppress invasion by its strong interaction with catenins, surrogating the role of E-cadherin in cell-cell adhesion [296]. Accordingly, in 2008, we have already shown that primary invasive breast carcinomas co-expressing E- and P-cadherin present a worse patient survival than carcinomas that express only one of the cadherins or that do not express any of these adhesion molecules [297]. These tumours have a decrease in membrane staining of p120-ctn and an increase in the cytoplasmic localization for this catenin. In pancreatic and ovarian cancer, it was shown that p120-ctn, once in the cytoplasm can activate Rho-GTPases (Ras homologue gene), Rac1 (Ras-related C3 botulinum toxin substrate 1) and Cdc42 (cell division cycle 42 Rho-family GTPase), altering the actin cytoskeleton polymerization and promoting cell motility [292, 298].

Importantly, P-cadherin aberrant expression is also found in other malignancies such as gastric, endometrial, colorectal and pancreatic carcinomas [271, 292, 299, 300]. Targeting P-cadherin in cancer may be a good therapeutical approach, since normal associated counterparts express very low levels of this molecule [271]. In fact, a novel and highly selective human monoclonal antibody against P-cadherin (PF-03732010) demonstrated anti-tumour and anti-metastatic activity in a panel of P-cadherin overexpressing tumour models, without significant secondary effects in mice [301, 302].

3.4.4 BREAST CSC MARKER

Several studies supports the hypothesis that cancers are propagated by a small population of cells that are present in the malignant tissue, with the ability to form a hierarchy similar to the one present in normal tissues [303]. These CSCs are able to proliferate, originating more stem-like cells, to exhibit resistance to current therapies and to remain quiescent during long periods of time. However, it is still not clear whether the CSC originates from the normal stem cells of the tissue that deregulate their self-renewal ability, or from normal mature cells or progenitor cells that acquired stem cell characteristics [303].

Although it is extremely important to define a universal phenotype for the breast CSCs, the existence of a single phenotype would be difficult mainly due to the high heterogeneity of this malignancy.

As described above, the aggressiveness and lack of target therapy exhibited by BLBC [304], draw much attention to the need of define a CSC phenotype for this poor-prognosis group of breast cancer. Recently, it has been demonstrated that the luminal progenitor of normal breast hierarchy is the cell of origin for this malignancy, since the induction of a *BRCA1* mutation in this cell was able to induce the formation of a tumour with basal phenotype [152, 305]. Since *CDH3* gene is repressed by BRCA1, it is likely that P-cadherin could be a good CSC marker of BLBC.

We have recently proposed P-cadherin as a breast CSC marker and a valuable target to be used in order to define the CSC phenotype of BLBC and the cell of origin of this disease [306]. We demonstrated that P-cadherin expression is able to promote stem like properties, such as mammosphere forming and 3D growth ability, being associated with the expression of CSC markers such as, CD44, CD49f and ALDH1. Additionally, cell lines with basal-like phenotype presented higher ALDEFLUOR^{bright} positivity comparing to luminal breast cancer cell lines [306]. We were able to establish the link between P-cadherin expression and the luminal progenitor and tumourigenic phenotype CD44^{+}CD24^{+}CD49f^{+} [152, 307].

Recently, we have also demonstrated that P-cadherin is responsible for the adhesion

of breast cancer cells to ECM, since its inhibition led to a significant decreased adhesion of cancer cells to the basement membrane substrate laminin, as well as to a major reduction in the expression of the laminin receptor α6β4 integrin [308]. Remarkably, the expression of this heterodimer was required for the invasive capacity and increased MFE induced by P-cadherin expression, which might explain the stem cell and invasive properties induced by P-cadherin in breast cancer cells, pointing to a new molecular mechanism that may be targeted to counteract the effects induced by this adhesion molecule [308].

Resistant to therapy regiments allows breast CSCs the ability to survive and persist in the tumour, being responsible for recurrence of the disease. Furthermore, it is accepted that breast CSC might be responsible for tumour relapses and further metastasis [217, 309]. Importantly, P-cadherin confers resistance to radiation in breast cancer cells. We have recently demonstrated that, when irradiated, P-cadherin-enriched cell population has increased ability to survive in anchorage-independent conditions [306]. Furthermore, we demonstrated that upon an apoptotic stimuli, decreased P-cadherin expression increases breast cancer cell death in a caspase-dependent mechanism [296].

CHAPTER II

Rationale and Aims

Poor prognosis presented by patients with BLBC has been associated, among other factors, with the glycolytic metabolism that these tumours present. Molecular adjustment to hypoxia and specific metabolic programs are responsible for the maintenance and expansion of breast cancer cells with stem-like properties, which are known to account for chemo and radioresistance of BLBC as well as to patient's relapses and distant metastasis. Despite the implications of the basal marker P-cadherin in patient's poor prognosis and the *in vitro* knowledge about the aggressiveness and stem-like properties of breast cancer cells with aberrant P-cadherin expression, nothing is known about the role of this protein in the adaptation of BLBC cells to adverse microenvironment conditions and to metabolic alterations occurring during tumour progression.

Main Aim

The main objective of this work was to evaluate the role of the basal epithelial marker P-cadherin in breast cancer cell metabolism.

Specific Aims

Using a series of primary invasive breast carcinomas and human breast cancer cell lines, the studies were performed in order to address the following specific aims:

I) To study the association between P-cadherin overexpression and the adapted molecular phenotype of breast cancer cells to hypoxia and to microenvironmental metabolic stress.

II) To evaluate if P-cadherin expression is affected by hypoxia and how its expression is connected to the molecular machinery involved in glycolytic and acid resistance phenotype of breast cancer cells.

III) To unravel the effect of P-cadherin expression in breast cancer cell's metabolism.

CHAPTER III

Materials and Methods

This chapter describes the materials and the methods used for all the data presented in the Results section.

Materials

Breast tumour samples

Primary invasive breast carcinomas used in this study were included in TMAs (Tissue Microarays) blocks, where representative areas of each breast tumour sample were carefully selected on the H&E-stained sections and marked on individual paraffin blocks. Two tissue cores (2 mm in diameter) were obtained from each specimen and precisely deposited into a recipient paraffin block using a TMA workstation (TMA builder 20010.02, Histopatholoy Ltd, Hungary).

A series of 473 formalin-fixed, paraffin-embedded invasive breast carcinomas was retrieved from the histopathology files of three Departments of Pathology: from University Hospital of the Federal University of Santa Catarina (Florianópolis, Brazil), from Hospital Divino Espírito Santo (Ponta Delgada, Portugal) and from a private Laboratory of Pathology (Araçatuba, Brazil). Data from clinical and pathological features of the tumours is summarized in **Table 1**. Immunohistochemical characterisation of this series was previously studied and described [283]. An independent series of 466 invasive breast carcinomas from the Department of Pathology, Hospital Xeral Cíes, Vigo, Spain, diagnosed between 1978 and 1992, was also used in part of this work. Detailed information about clinical and molecular characteristics is described by Vieira *et al.* [306]. The studies using this material were conducted under the national regulative law for the handling of biological specimens from tumour banks, being the samples exclusively available for research purposes in retrospective studies.

Table 1. Clinical, pathological and immunohistochemical characteristics of the 473 primary invasive breast carcinomas. Characterization of the breast cancer series concerning age of the patients at the diagnosis, tumour size, lymph-node metastasis, histological grade, molecular subtype, ER, PgR, HER2 and Ki67 status, as well as expression of P-cadherin, HIF-1α, GLUT1, CAIX, MCT1, MCT4 and CD147.

Clinico-pathological and molecular characteristics	Age (years)	Mean	56.12	
		Min	27	
		Máx	89	
		Missing	2	
	Tumour size (mm)	Mean	31.6	
		Min	5	
		Máx	150	
		Missing	243	
			Frequency (n)	(%)
	Lymph-node metastasis	Positive	204	51
		Negative	196	49
		Total	400	100
		Missing	73	-
	Histological grade	I	155	33.2
		II	200	42.8
		III	112	24
		Total	467	100
		Missing	6	-
	Molecular subtypes	Luminal A	262	57.3
		Luminal B	14	3.1
		HER2 OE	56	12.3
		Basal	83	18.2
		Unclassified	42	9.2
		Total	457	100
		Missing	16	-
	ER	Positive	275	58.4
		Negative	196	41.6
		Total	471	100
		Missing	2	-
	PR	Positive	177	37.9
		Negative	290	62.1
		Total	467	100
		Missing	6	-
	HER2	Positive	69	14.9
		Negative	393	85.1
		Total	462	100
		Missing	11	-
	Ki67	>20	98	39.5
		<20	150	60.5
		Total	248	100
		Missing	225	-
Biomarkers	P-cadherin	Positive	145	31
		Negative	323	69
		Total	468	100
		Missing	5	-
	HIF-1α	Positive	104	33
		Negative	211	67
		Total	315	100
		Missing	158	-
	GLUT1	Positive	140	42.8
		Negative	187	57.2
		Total	327	100
		Missing	146	-
	CAIX	Positive	66	20.9
		Negative	250	79.1
		Total	316	100
		Missing	157	-
	MCT1	Positive	106	26
		Negative	301	74
		Total	407	100
		Missing	66	-
	MCT4	Positive	69	16.5
		Negative	350	83.5
		Total	419	100
		Missing	54	-
	CD147	Positive	24	11.1
		Negative	193	88.9
		Total	217	100
		Missing	256	-

Cell culture

Human breast cancer cell lines were obtained as follows: BT20 and MDA-MB-468 were acquired from American Type Culture Collection (Manassas, VA, USA), SUM149 was kindly provided by Dr. Stephen Ethier (University of Michigan, USA) and MCF-7/Az was kindly given by Prof. Marc Mareel, Ghent University, Belgium. MCF-7/Az cell line was retrovirally stable transduced to encode P-cadherin (MCF-7/Az.Pcad cell line), as described earlier [241]. MCF-7/Az.Mock cell line, encoding only EGFP, was used as a control. Cells were routinely maintained at 37°C and 5% CO_2 in the following media (Invitrogen Ltd, UK): DMEM for BT20 and for MDA-MB-468 and 50% DMEM/50% Ham-F12 for SUM149 and MCF-7/Az. In BT20 and MCF-7/Az, the media contained 10% heat-inactivated fetal bovine serum, FBS, (Greiner bio-one, Belgium) and in SUM149 cell line, media was supplemented with 5% FBS, 5 µg/ml of insulin and 1 µg/ml of hydrocortisone (Sigma-Aldrich, USA). All media were supplemented with 100 IU/ml penicillin and 100 mg/ml streptomycin (Invitrogen Ltd, UK).

Primary Antibodies

The following primary anti-human antibodies were used for western blot (WB), immunohistochemistry (IHC) and flow cytometry (FC) against: P-cadherin [clone 56, BD Transduction Biosciences, USA; diluted 1:500 (WB) and 1:50 (IHC)] and APC-conjugated P-cadherin, R&D, USA; diluted 1:10 (FC)], HIF-1α [clone 54, BD Transduction Biosciences, USA; diluted 1:500 (WB) and 1:50 (IHC)], CAIX [ab15086, AbCam, Cambridge, UK; diluted 1:1000 (WB), 1:2000 (IHC) and 1:10 (FC)], GLUT1 [ab15309, AbCam, UK; diluted 1:400 (WB), 1:500 (IHC) and 1:10 (FC)], MCT1 [AB3538P, Chemicon International, USA; diluted 1:200 (IHC)], MCT4 [AB3316P, Chemicon International, USA; diluted 1:100 (IHC)], CD147 [18-7344, Zymed Laboratories Inc., USA; diluted 1:750 (IHC)], CD44 [clone 156-3C11; Cell Signalling Technology, USA; diluted 1:100 (IHC)], CD49f [HPA001814; Sigma-Aldrich, USA, diluted 1:10 (IHC)], SOD1 [Cell Signalling Technology, USA; diluted 1:2000 (WB)], SOD2 [HPA001814, Sigma-Aldrich; diluted 1:2500 (WB)], p-PDH Ser293 [ab177461, AbCam, UK; diluted 1:2000 (WB)], PDH E1 [clone 9H9AF5, MitoSciences, USA;

diluted 1:1000 (WB)], PDK2 [HPA008287, Sigma-Aldrich, USA; diluted 1:1000 (WB)], GAPDH [0411, Santa Cruz Biotechnologies, USA; diluted 1:10000 (WB)] and β-actin [clone I-19, Santa Cruz Biotechnologies, USA; diluted 1:1000 (WB)].

METHODS

Immunohistochemistry

The immunohistochemical assays were performed with specific antibodies for P-cadherin, HIF-1α, GLUT1, CAIX, MCT1, MCT4, CD147, CD44 and CD49f. Details about experimental procedures, primary antibodies, antigen retrieval detection systems and scoring are described elsewhere [71, 99, 283, 306]. Specifically, HIF-1α immunohistochemistry was performed using CSA, Catalyzed Signal Amplification System (DAKOCytomation, USA), according to manufacturer's instructions. Reactions were independently evaluated by two pathologists. All the proteins showed membrane staining, consistent with their cellular function, except for HIF-1α, which presented a nuclear pattern of expression and was considered positive whenever any strong and dark nuclear staining was observed. For the different antibodies studied, some samples could not be evaluated for the 473 cases of the series due to TMA's cores missing or to insufficient representation of the tumour in the TMA core. Statistical analysis was performed by SPSS statistics 17.0 software (SPSS Inc., USA). χ^2 test and contingency tables were used to determine associations between groups and the results were considered statistically significant when the p-value was lower than 0.05.

cDNA Microarrays

cDNA Microarray procedure and analyses are described in previous works from our group [310, 311]. The complete array data from MCF-7/Az and BT20 models can be viewed in Gene Expression Omnibus database (GEO accession No GSE54319) and in the ArrayExpress microarray database (accession No E-MEXP-3329), respectively. Differences in gene expression ($p<0.01$ and fold-change>2) between the two conditions in both models were functionally annotated using DAVID [Database for

Annotation, Visualization and Integrated Discovery [312]]. Using DAVID's default parameters, several significantly enriched biological pathways/gene ontology terms were obtained which are further described in the Results section.

Hypoxia

Breast cancer cells were plated in flasks in order to be at approximately 40% of confluence and to avoid overconfluence at the end of the 5th day of the experiment. After the adhesion of the cells overnight, the flasks were placed in the incubator at 1% of oxygen and 5%CO_2, at 37°C. In the end of each time point, cells were immediately placed on ice, washed twice in PBS (phosphate-buffered saline) and lysed with catenin lysis buffer (See "Protein extraction and western blot analysis" in this section) in the shortest time possible in order to avoid oxygen-induced HIF-1α degradation.

$CoCl_2$ treatment

Breast cancer cells were plated in T25 flasks or in coverslips, for FACS or western blot and for immunofluorescence, respectively. After 24hours, cells were treated with 200μM $CoCl_2$ (Cobalt(II) chloride hexahydrate, Sigma-Aldrich, USA) for 4 hours.

Protein extraction and western blot analysis

Protein lysates were prepared from cells, using catenin lysis buffer [1% (v/v) Triton X-100 and 1% (v/v) NP-40 (Sigma-Aldrich, USA) in PBS] supplemented with 1:7 proteases inhibitors cocktail (Roche Diagnostics GmbH, Germany) for 10 min, at 4°C. Cell lysates were mixed with a vortex and centrifuged at 14000 rpm at 4°C, during 10 min. Supernatants were collected and protein concentration was determined using the Bradford assay (BioRad Protein Assay kit, USA). Proteins were dissolved in sample buffer [Laemmli with 5% (v/v) 2-β-mercaptoethanol and 5% (v/v) bromophenol blue] and boiled for 5 min at 95°C or at 65°C (for GLUT1 staining). Samples were separated by SDS–PAGE and proteins were transferred into nitrocellulose membranes [Amersham Hybond enhanced chemiluminescence (ECL)]. For immunostaining, membranes were blocked with 5% (w/v) non-fat dry milk in PBS containing 0.5% (v/v) Tween20 and incubated during 1 hour with anti-P-cadherin,

anti-GAPDH and anti-β-actin, two hours with anti-CAIX and anti-GLUT1 and overnight for anti-HIF-1α, SOD1, SOD2, SOD2-k68, p-PDH, PDH and anti-PDK. After washes with PBS-Tween20, membranes were incubated with HRP-conjugated anti-mouse, goat or rabbit secondary antibodies (Santa Cruz Biotechnologies, USA) diluted 1:2000 for one hour. Proteins were then detected using ECL reagent (Amersham, USA) as a substrate. Quantity One software (BioRad, USA) was used for quantification of the differences in protein expression comparing with GAPDH expression.

Flow Cytometry and Cell Sorting

For flow cytometry analysis, cells were harvested with versene/0.48mM EDTA (Invitrogen, UK), washed with PBS supplemented with 0.5% FBS and re-suspended in the stain buffer (2mM EDTA and 0.5% bovine albumin in PBS). Single cell suspension was labelled with APC-conjugated P-cadherin, GLUT1 and CAIX antibodies. Cells transfected with the control siRNA and with *CDH3* siRNA were doubled stained either with P-cadherin and GLUT1 or CAIX antibodies. A live-dead stain (Invitrogen, UK) and the primary and secondary antibodies were incubated at 4°C, in the dark, for 15 minutes. Secondary Alexafluor-488-conjugated goat anti-rabbit IgG (Invitrogen, UK) was used in a 1:100 dilution. The labelled cells were then washed in the stain buffer and analysed on a FACS Canto-II (BD Biosciences, USA).
For the sorting experiments, the subpopulations of SUM149 and BT20 breast cancer cells were selected according to P-cadherin expression (highest and lowest 20% expressing cells). Cells were sorted using BD Influx or FACS ARIA-II (BD Biosciences) and collected into 10% Hanks buffered solution (Invitrogen, UK). The purity of sorted populations was 80-95%. In addition, a further sample was also collected of cells passed through the laser under pressure, but not sorted, to act as a control for the effect of the pressure on the cells.

Immunofluorescence and confocal microscopic analysis

Cells were cultured on glass coverslips and 24 hours later they were treated either with 200μM of $CoCl_2$ or with the respective vehicle (Ethanol) during 4 hours. After

that, cells were fixed with 4% paraformaldehyde (20 minutes), treated with NH4Cl (50mM) for 10 minutes, washed with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes, at room temperature. Unspecific reactions were blocked by incubation of cells with blocking solution (5% BSA in PBS-tween 0,5%) during 30 minutes. Cells were then stained with the primary antibodies, followed by incubation in the dark with Alexa488 or Alexa-594-conjugated secondary IgGs (Dako Cytomation, Carpinteria, CA) in a 1:500 dilution. Primary and secondary antibodies were diluted in blocking solution. Each sample was mounted with Vectashield (Vector Laboratories, Inc, Burlingame, CA) containing 4,6-diamidine-2-phenylindolendihydrochrolide (DAPI) and visualized with Leica SP5 confocal microscope (Leica Microsystems GmbH, Germany). Volume of cells of both conditions was acquired by Z-stack measurements.

siRNA transfection

Gene silencing was performed with validated small interfering RNAs (siRNA), specific for *CDH3* (50nM, Hs_*CDH3*_6), HIF-1α (50nM, Hs_HIF1A_5), *GLUT1* (100nM Hs_*SLC2A1*_2) and *CAIX* (50nM, Hs_*CA9*_2). All siRNAs were from Qiagen (USA). Transfections were carried out using Lipofectamine 2000 (Invitrogen, UK), according to manufacturer's recommended procedures. After incubation for 5 minutes, the siRNA and Lipofectamine 2000 solutions were mixed, incubated for additional 20 minutes and added to cell culture medium. A scrambled siRNA sequence, with no homology to any gene, was used as a negative control (Qiagen, USA). Gene inhibition was evaluated after 48 hours of cell transfection for *CDH3,* HIF-1α and *GLUT1* and after 72 hours for *CAIX*.

RNA extraction and qRT-PCR

RNA extraction was performed using RNeasy Mini Kit (Qiagen, USA) and cDNA was synthesized using the Omniscript Reverse Transcription kit (Qiagen, USA), following the manufacturer's instructions. Quantitative-Real-Time-PCR (qRT-PCR) reaction was performed with TaqMan Gene Expression Assays (Applied Biosystems, USA), using gene-specific IDT probes (Integrated DNA Technologies, Inc., USA): *CDH3*

(Hs.PT.51.5028751), *GLUT1* (Hs.PT.47.19044492.g), *CAIX* (Hs.PT.47.1458063.g), and *GAPDH* (Hs.PT.39a.22214836). Analysis was performed with the ABI PRISM 7700 Sequence Detection System Instrument and software (Applied Biosystems, USA), following the manufacturer's recommendations. The internal standard human *GAPDH* was used to normalize cDNA quantity. Data was analysed by the comparative 2(-ΔΔCT) method [313]. For all data comparisons, the Student's t-Test was used (two tailed, unequal variance). All reactions were done in triplicate and the results presented as mean of the values from three or more independent experiments.

Mammosphere forming efficiency (MFE) assay

After the 72h of the siRNA transfection, cells were enzymatically harvested and manually disaggregated with a 25-gauge needle to form a single-cell suspension and resuspended in cold PBS. Cells were plated at 500/cm^2 in non-adherent culture conditions, in flasks coated with 1.2% poly(2-hydroxyethylmethacrylate)/95%ethanol (Sigma-Aldrich, USA) and allowed to grow for 5 days, in DMEM/F12 containing B27 supplement (Invitrogen Ltd, UK) , and 500 ng/ml hydrochortisone Sigma-Aldrich, USA), 40 ng/ml insulin (Sigma-Aldrich, USA), 20 ng/ml EGF)Sigma-Aldrich, USA) in a humidified incubator at 37°C and 5% (v/v) CO2. Mammosphere forming efficiency was calculated as the number of mammospheres (≥50 μm) formed divided by the number of cells plated, being expressed as a percentage.

OCR and ECAR measurements

Oxidative phosphorylation and glycolysis was accessed using a Seahorse XF96 Extracellular Flux Analyzer (Seahorse Bioscience, USA). The oxygen-sensitive fluorophore measures OCR (oxygen consumption rate) during OXPHOS and the pH-sensitive fluorophore measures the extracellular acidification rate (ECAR), which is proportional to the rate of lactate production by glycolysis.

Twenty-four hours after the transfection, 40000 cells were seeded in a XF96 cell culture microplates and allowed to adhere overnight at 37°C and 5%CO_2, reaching 80-90% confluence in the next day. The XF sensor cartridge was calibrated overnight

with XF Calibrant Solution (Seahorse Bioscience, USA) in a 0% CO_2 incubator. In the following day, cells were equilibrated in unbuffered *XF Base Medium* (Seahorse Bioscience, USA), for 1 hour at 0% CO_2 prior to analysis. This *XF Base medium* was supplemented with glucose (3,15 g/dL of glucose for SUM149 and with 4,5g/dL and MDA-MB-468), 2mM L-glutamine (Invitrogen Ltd, UK) and 1mM pyruvate (Invitrogen Ltd, UK). Bioenergetics measurements were performed by the injection of oligomycin to a final concentration between 3μg/mL. Maximal FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone) effect was observed with two injections to final concentrations between 1μM and 3μM. Experiments were performed independently for at least three times, each one with eight technical replicates. Oligomycin A and FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone) were obtained from Sigma-Aldrich, USA.

After the XF assay, the number of cells in each well was measured with Vibrant dye (Life Technologies, USA). The dye was incubated at 1μM during 15 minutes and the number of cells was determined by object counting in *IncuCyte* incubator and using *IncuCyte* Analyser V1 software.

ATP analysis

ATP measurements were performed using CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, USA), according to manufacturer's instructions. $5X10^4$ of control and *CDH3* siRNA transfected cells were resuspended in 80μL and plated in a clear bottom 96 well plate. Cells were then treated with PBS (control), oligomycin A (10μg/mL) and FCCP (30μM), alone or in combination with 100μM sodium iodoacetate (Sigma-Aldrich, USA). Following 1 hour of incubation with the reagents at 37°C and 5% CO_2, 100μL of Cell Titer Glo reaction mix were added to each well for a final volume of 200μL. Plates were then analysed for luminescence in Labsystems Luminoskan Ascent Microplate luminometer (Thermo Scientific, USA). Measurements were performed 1 hour after the cells were plated, as well as after 24 hours of incubation in normoxia or hypoxia. Experiments were performed independently for at least three times, each one with five technical replicates.

ROS assay

After incubation for 4 to 6 hours with transfection reagents, cells were washed twice with PBS and the medium was changed to DMEM/F12 with no phenol-red (Invitrogen Ltd, UK), without serum nor antibiotics, until the endpoint of transfection (~48 hours). Cells were then washed and DCHF-DA (Sigma-Aldrich, USA) was added to a final concentration of 10μM for 30 minutes at 37°C. After washing twice, 200μL of PBS was added to each well and fluorescence was read on λ_{ex}=485nm and λ_{em}=535nm.

Superoxide dismutase activity

Superoxide dismutase activity in breast cancer cell extracts was determined by native PAGE staining according to Flohé and Otting [314]. After electrophoresis of the cells extracts under native conditions, the gel was incubated in the dark for 30 minutes in a solution containing 2.5 mM NBT (nitroblue tetrazolium) followed by a 20 minutes incubation in developing solution, composed of 36 mM phosphate buffer, pH 7.8, 28 mM TEMED, and 2.8×10^{-5}M riboflavin. The gel was rinsed in dH_2O and kept under shaking and light (60 W) until bands became visible. The reaction was stopped in a 7.5% acetic acid solution.

Apoptosis assay

Twenty-four hours after the transfection, 12 000 cells were seeded in a 96 well plate. When cells were adherent, a caspase 3/7 substrate (NucView™ 488, Essen Bioscience, USA) was added to a final concentration of 5μM and the plate was placed in the *IncuCyte* incubator (Essen Bioscience, USA) in order to be monitored at each 3 hours. Phase contrast and fluorescence data were obtained at each time point. Data analysis of AUC (Area Under Curve) of apoptosis measurements was performed using *IncuCyte Analyzer V1* software. Experiments were performed independently for at least three times, each one with ten technical replicates.

Mitotracker CMXRos

Mitotracker CMXRos (Invitrogen Ltd, UK) was used to evaluate mitochondrial membrane potential by FACS analysis. Control and *CDH3* siRNA transfected cells

were incubated with 250nM of Mitotracker CMXRos during 30 minutes. After wash twice with PBS, cells were harvested with versene/0.48mM EDTA (Invitrogen, UK), washed with PBS supplemented with 0.5% FBS and re-suspended in the stain buffer, as described above (See “Flow Cytometry and Cell Sorting” in this section). Single cell suspension was labelled with APC-conjugated P-cadherin (1:10) during 15 minutes, washed in the stain buffer and analysed on a FACS Canto-II (BD Biosciences, USA).

Statistical analysis

Results are representative of three or more independent experiments. Quantifications are expressed as mean ± SEM of the biological replicates considered. Statistical analyses were performed using Office Excel 2010 (Microsoft Corporation, Reading, UK). All statistical tests were two-sided and considered as significant when *P* value was less than 0.05.

CHAPTER IV

Results

I. ASSOCIATION BETWEEN P-CADHERIN OVEREXPRESSION AND THE ADAPTED MOLECULAR PHENOTYPE OF BREAST CANCER CELLS TO HYPOXIA AND TO MICROENVIRONMENTAL METABOLIC STRESS

The main purpose of this part of the work was to search for evidences that would support the association between P-cadherin aberrant expression and hypoxic conditions, as well as metabolic alterations observed in breast cancer. For that, we examined online available gene expression databases and used bioinformatics predictive tools. Additionally, we also analysed the mRNA profile of two distinct breast cancer cell models after P-cadherin modulation, in order to find if this could affect the expression of genes implicated in tumour microenvironmental-induced cellular response. Finally, we also evaluated the expression of P-cadherin and hypoxic, glycolytic and acidosis markers in a series of primary invasive breast carcinomas.

Ia) Bioinformatics evidences of P-cadherin modulation by microenvironmental conditions in breast cancer

The analysis of an online available gene expression profile (GEP) (E-GEOD-9649) [315] in Gene Expression Atlas [316] revealed that *CDH3* gene is indeed altered in human mammary epithelial cells (HMEC) when exposed to different microenvironmental conditions observed in solid tumours. Comparing to normoxia conditions, *CDH3* was found to be upregulated when HMEC were cultured for 24 hours in hypoxia (2% of oxygen) at neutral pH (**Figure 13A**). Moreover, *CDH3* levels were also upregulated in lactic acidosis (25mM of lactic acid at pH 6.7) and downregulated in lactosis conditions (25mM of sodium lactate, neutral pH) (**Figure 13B**).

Interestingly, using bioinformatics prediction tools [317, 318], we were also able to recognize a putative binding site for HIF-1 transcription factor, positioned in a CpG island within the *CDH3/P-cadherin* promoter [319].

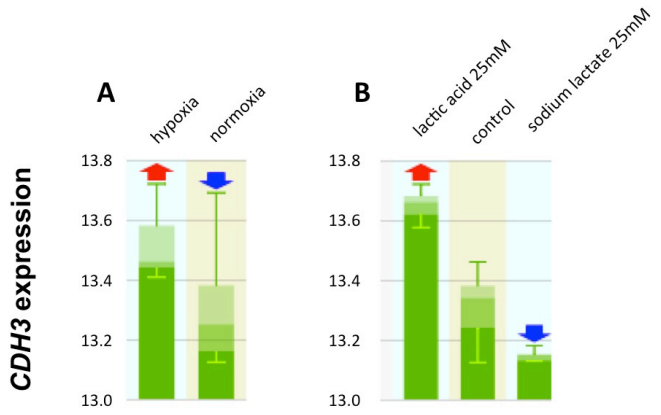


Figure 13. *CDH3* expression alterations in different microenvironmental conditions observed in solid tumours. Gene expression analysis of Array Express E-GEO-9649 shows that *CDH3* is enriched in HMEC cultured during 24 hours in hypoxia comparing to normoxia culture conditions. Lactic acidosis also increases *CDH3* levels in HMEC, while lactosis induces a decrease of *CDH3* expression.

Ib) P-cadherin is associated with the expression of genes related to the response to oxygen levels and metabolic processes in breast cancer cells

The analysis of cDNA microarrays data, obtained from previous experiments performed in our research group, suggested that the expression of P-cadherin is able to induce alterations in the expression of genes implicated in cellular response to oxygen, nutrients, as well as in metabolic changes of breast cancer cells.

The mRNA profile after P-cadherin modulation was evaluated in two different models: the MCF-7/Az luminal breast cancer cell model, where P-cadherin was constitutively overexpressed, and the BT20 BLBC cell model, where P-cadherin expression was silenced using a specific *CDH3* siRNA. Gene ontology terms, specific genes and fold enrichment are summarized in **Table 2.**

Table 2. Gene ontology terms, genes and fold change of the differentially expressed genes of MCF-7/Az and BT20 models with P-cadherin overexpression and silencing, respectively.

Model	Gene Ontology Term	Genes	Fold change	p-value
MCF-7/Az (mock vs P-cadherin)	Glutathione metabolism	*GSTM1, GGT6, GSTM3, OPLAH, GPX6, GPX3*	33.7	**0.0308**
	Regulation of cyclic nucleotide metabolic process	*GUCA1A, P2RY11, ADORA2B, ADORA2A, APOE, GRM7, ADCY5, S1PR4, NTRK1, EDN1, GUCY1A3, TIMP2*	3.3	**9.49E+11**
	Carboxylic acid transport	*CPT1B, SLC22A16, SLC16A5, CD36, SLC6A7, SLC6A20, SLC7A2, BDKRB2, SLC7A5, PDZK1*	2.1	**0.0456**
	Response to nutrient levels	*TNFRSF11B, CCK, GATM, CD44, FOXA3, IGFBP7, SOX2, MGP, ANGPT1, ALOX5, TIMP3*	17.5	0.0992
	Response to oxygen levels	*GPR182, CDKN1A, PYGM, CXCR4, EDN1, HIF3A, APOLD1, ANGPT1, SERPINA1*	2.0	0.0815
BT20 (siCtr vs si*CDH3*)	Glycerol metabolic process	*GPD1, DGAT2, GK5*	10.4	**0.0327**
	Glucose metabolic process	*AKT1, GPD1, NISCH, PFKL, NPY1R, DHTKD1*	3.1	**0.0421**
	Fructose 6-phosphate metabolic process	*PFKL, GFPT1*	22.9	0.0838
	Apoptotic mitochondrial changes	*AKT1, CASP7, IFI6*	7.7	0.0564
	Regulation of lipid metabolic process	*AKT1, RAC1, SERPIN3, ATP1A1, PRKAA2*	3.6	0.0508

In MCF-7/Az model, the mRNA profile identified 867 genes whose expression was deregulated in MCF-7/Az.P-cadherin cells comparing to the control MCF-7/Az.mock cells ($p<0.01$; fold-change>2). Interestingly, we found that genes involved in glutathione metabolism, which can be responsible for regulation of antioxidant balance and detoxifying processes, were deregulated in breast cancer cells with overexpression of P-cadherin in comparison to the control cells (fold change=33.7; *p=0.0308*); in addition, genes associated with cyclic nucleotide metabolic processes (fold change=3.3; $p=9.49X10^{-9}$) and with the transport of carboxylic acids (fold change=2.1; *p=0.0456*) were also found to be deregulated by P-cadherin overexpression (**Table 2**). Moreover, although not statistically significant, we also observed alterations of about 17.5-fold change in gene expression linked to the

response to nutrient levels and a 2-fold change in the response to oxygen levels (**Table 2**).

Concerning the BT20 breast cancer cell model, as previously described [310], the profile identified 237 genes, whose expression was deregulated ($p<0.01$; fold-change>2) in cells with *CDH3* siRNA comparing to the control cells. Again, we could observe that the GEP involving several metabolic processes was distinct in cells with P-cadherin knockdown comparing the one presented by the control cells. Interestingly, we observed that P-cadherin downregulation interferes with the glycerol (fold change=10.4; *p=0.0327*) and glucose metabolic processes (fold change=3.1; *p=0.0421*), (**Table 2**). In addition, we also found that genes involved in lipid metabolism (fold change=3.6; *p=0.0508*), apoptotic mitochondrial changes (fold change=7.7; *p=0.0564*), as well as with fructose 6-phosphate metabolism, were also being deregulated by P-cadherin expression in breast cancer cells, although the difference was not statistically significant (**Table 2**).

Although these results need further validation, they strongly suggests that, in normoxia conditions, P-cadherin overexpression in breast cancer cells is interfering with the expression of genes involved in the response to oxygen levels, as well as with metabolic alterations observed in breast cancer.

Ic) P-cadherin overexpression is significantly associated with the expression of hypoxic, glycolytic and acidosis biomarkers in primary invasive breast carcinomas

In a large series of invasive breast carcinomas (n=473), previously classified for molecular subtypes [283], immunohistochemistry staining was performed for P-cadherin, HIF-1α, GLUT1, CAIX, MCT1, MCT4, and CD147 **(Figure 14)**. Membrane P-cadherin expression was found in 145/468 (31%) of the cases. Nuclear HIF-1α was considered positive in 104/315 (33%) carcinomas. Concerning the membrane expression of GLUT1, CAIX, MCT1, MCT4 and CD147, we observed 140/327 (42.8%), 66/316 (20.8%), 106/407 (26%), 69/419 (16.5%) and 24/217 (11%) positive

cases, respectively. Membrane GLUT1 and CAIX expression was frequently detected in peri-necrotic tumour areas.

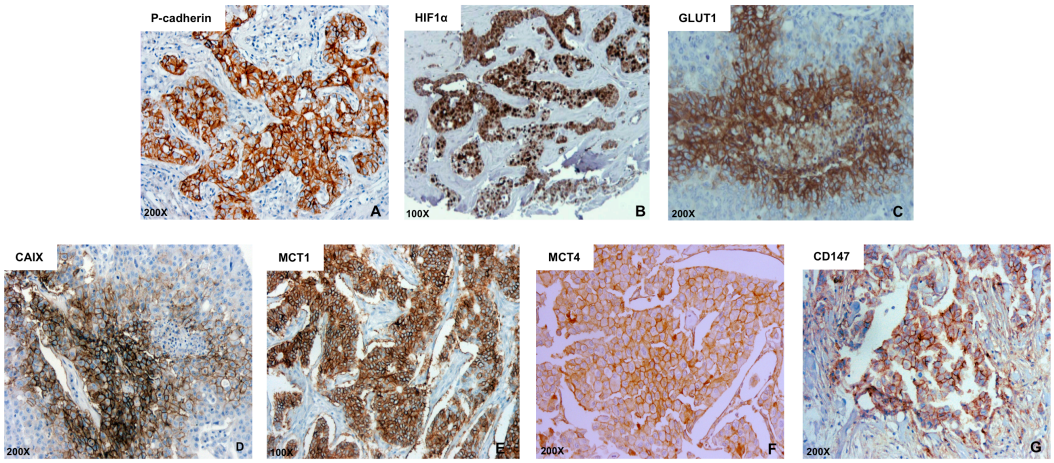


Figure 14. Breast cancer immunoreactivity for P-cadherin, hypoxia, glycolytic and acid resistant biomarkers. Immunohistochemical staining for P-cadherin (A), HIF-1α (B), GLUT1 (C), CAIX (D), MCT1 (E), MCT4 (F) and CD147 (G) expression in primary invasive breast carcinomas. Images A, C, D, F and G are in 200x magnification; B and E are in 100X magnification.

The association between the expression of each one of these markers with the classical breast cancer prognostic factors (**Table 3**), as well as with the molecular subtypes and biomarkers ER, PgR, HER2 and Ki67, was evaluated (**Table 4**). As previously reported, P-cadherin expression was significantly associated with high-grade carcinomas (*p<0.0001*), HER2-overexpressing and basal-like molecular subtypes (*p<0.0001*), ER and PgR negativity (*p<0.0001*), high expression of HER2 (*p<0.0001*), as well as with high Ki67 (*p=0.0141*). Accordingly, HIF-1α expression was also associated with grade III (*p<0.0001*) and high proliferative (*p=0.0197*) tumours. Concerning the expression of GLUT1, CAIX, MCT1 and CD147, all have been significantly associated with high-grade (*p<0.001*), basal-like (*p<0.001*), ER and PgR negative tumours (*p<0.05*); absence of lymph node metastasis was more frequently observed in MCT1 expressing tumours (*p=0.0223*) and CAIX expression was associated with an increased tumour size (*p=0.0005*). Additionally, the

expression of GLUT1, MCT1 and CD147 was associated with high proliferation indexes measured by Ki67 expression (*p=0.0339*, *p=0.0297*, *p=0.0179*, respectively).

Table 3. Association of P-cadherin, HIF-1α, GLUT1, CAIX, MCT1, MCT4 and CD147 expression with the classic prognostic factors in breast cancer.

		P-cadherin			HIF-1α			GLUT1			CAIX			MCT1			MCT4			CD147		
		Positive	Negative	p	Positive	Negative	p	Positive	Negative	p	Positive	Negative	p	Positive	Negative	p	Positive	Negative	p	Positive	Negative	p
Histological Grade	I	21 (14.5%)	133 (41.9%)		30 (28.8%)	91 (44.2%)		35 (25.7%)	84 (45.4%)		13 (20.3%)	100 (40.7%)		29 (28.4%)	99 (33.1%)		28 (40.6%)	109 (31.7%)		0 (0%)	44 (22.8%)	
	II	57 (39.3%)	140 (44.2%)	***<0.0001***	39 (37.5%)	88 (42.7%)	***<0.0001***	53 (39%)	76 (41.1%)	***<0.0001***	23 (35.9%)	101 (41%)	***<0.0001***	35 (34.3%)	142 (47.5%)	***0.0011***	24 (34.8%)	153 (44.5%)	*0.2663*	6 (25%)	96 (49.7%)	***<0.0001***
	III	67 (46.2%)	44 (13.9%)		35 (33.7%)	27 (13.1%)		48 (35.3%)	25 (13.5%)		28 (43.8%)	45 (18.3%)		38 (37.3%)	58 (19.4%)		17 (24.6%)	82 (23.8%)		18 (75%)	53 (27.5%)	
Lymph-node metastasis	Positive	63 (47.7%)	134 (50.2%)	*0.6438*	44 (50.6%)	90 (51.1%)	*0.9317*	65 (53.7%)	72 (45%)	*0.1477*	32 (57.1%)	102 (46.8%)	*0.1668*	36 (39.6%)	138 (53.5%)	***0.0223***	31 (57.4%)	147 (48.7%)	*0.2372*	9 (37.5%)	90 (52.9%)	*0.1566*
	Negative	69 (52.3%)	133 (49.8%)		43 (49.4%)	86 (48.9%)		56 (46.3%)	88 (55%)		24 (42.9%)	116 (53.2%)		55 (60.4%)	120 (46.5%)		23 (42.6%)	155 (51.3%)		15 (62.5%)	80 (47.1%)	
Tumour Size	Mean±SE (n)	33.1±2.5 (81)	30.8±1.7 (145)	*0.4375*	34.8±3.2 (60)	36.1±2.9 (67)	*0.764*	30±2.7 (54)	29.3±2.1 (67)	*0.8581*	42.2±6.4 (21)	27.2±1.4 (98)	***0.0005***	35.5±3.6 (38)	31.2±1.7 (165)	*0.2617*	24.1±2.7 (15)	32.5±1.6 (186)	*0.1454*	29.4±3.5 (22)	32.1±1.6 (180)	*0.5906*

Table 4. Association of P-cadherin, HIF-1α, GLUT1, CAIX, MCT1, MCT4 and CD147 with molecular subtypes and biomarkers ER, PgR, HER2 and Ki67 in breast cancer.

		P-cadherin			HIF-1α			GLUT1			CAIX			MCT1			MCT4			CD147		
		Positive	Negative	p	Positive	Negative	p	Positive	Negative	p	Positive	Negative	p	Positive	Negative	p	Positive	Negative	p	Positive	Negative	p
Molecular Subtypes	Luminal A	35 (35.4%)	223 (69.5%)		49 (48.6%)	122 (59.5%)		73 (53.3%)	121 (62.9%)		26 (40%)	162 (66.7%)		48 (46.2%)	171 (58.5%)		44 (65.6%)	186 (54.4%)		4 (18.2%)	110 (58.8%)	
	Luminal B	3 (3.0%)	11 (3.4%)		6 (5.9%)	6 (2.9%)		3 (2.2%)	4 (2.2%)		0 (0%)	7 (2.9%)		3 (2.9%)	10 (3.4%)		3 (4.5%)	10 (2.9%)		0 (0%)	7 (3.7%)	
	HER2 OE	38 (38.4%)	18 (5.6%)	***<0.0001***	16 (15.8%)	27 (13.2%)	*0.076*	12 (8.8%)	20 (11%)	***0.0001***	10 (15.4%)	20 (8.2%)	***<0.0001***	8 (7.7%)	44 (15.1%)	***<0.0001***	3 (4.5%)	52 (15.2%)	***0.032***	4 (18.2%)	28 (15%)	***<0.0001***
	Basal	23 (23.2%)	27 (8.4%)		23 (22.8%)	27 (13.2%)		41 (29.9%)	17 (9.4%)		24 (36.9%)	33 (13.6%)		38 (36.5%)	37 (12.7%)		15 (22.4%)	59 (17.3%)		12 (54.5%)	29 (15.5%)	
	Unclassified	0 (0%)	42 (13.1%)		7 (6.9%)	23 (11.2%)		8 (5.8%)	19 (10.5%)		5 (7.7%)	21 (8.6%)		7 (6.7%)	30 (10.3%)		2 (3%)	13 (7%)		2 (9.1%)	13 (7%)	
ER	Positive	37 (25.9 %)	233 (72.1%)	***<0.0001***	56 (30.6%)	46 (35.4%)	*0.3736*	76 (54.7%)	123 (65.8%)	***0.0421***	25 (37.9%)	168 (67.5%)	***<0.0001***	50 (47.6%)	182 (60.5%)	***0.022***	48 (70.6%)	194 (55.4%)	***0.0205***	5 (20.8%)	118 (61.5%)	***0.0002***
	Negative	106 (74.1%)	90 (27.9%)		127 (69.4%)	84 (64.6%)		63 (45.3%)	64 (34.2%)		41 (62.1%)	81 (32.5%)		55 (52.4%)	119 (39.5%)		20 (29.4%)	156 (44.6%)		19 (79.2%)	74 (38.5%)	
PgR	Positive	24 (16.8%)	150 (46.9%)	***<0.0001***	34 (33.7%)	82 (39%)	*0.3579*	41 (29.3%)	87 (46.5%)	***0.016***	19 (28.8%)	106 (42.4%)	***0.0443***	30 (28.3%)	121 (40.6%)	***0.0245***	30 (43.5%)	129 (36.5%)	*0.2402*	2 (8.3%)	78 (40.6%)	***0.002***
	Negative	119 (83.2%)	170 (53.1%)		67 (66.3%)	128 (61%)		99 (70.7%)	100 (53.5%)		47 (71.2%)	144 (57.6%)		76 (71.7%)	177 (59.4%)		39 (56.5%)	224 (63.5%)		22 (91.7%)	114 (59.4%)	
HER2	Positive	41 (28.7%)	28 (8.9%)	***<0.0001***	22 (21.6%)	32 (15.7%)	*0.2032*	15 (10.9%)	23 (12.5%)	*0.6705*	10 (15.4%)	26 (10.6%)	*0.2857*	10 (9.7%)	54 (18.4%)	***0.0397***	6 (9%)	61 (17.8%)	*0.0738*	4 (17.4%)	35 (18.4%)	*0.904*
	Negative	102 (71.3%)	287 (91.1%)		80 (78.4%)	172 (84.3%)		122 (89.1%)	161 (87.5%)		55 (84.6%)	219 (89.4%)		93 (90.3%)	240 (81.6%)		61 (91%)	282 (82.2%)		19 (82.6%)	155 (81.6%)	
Ki67	High	43 (48.9%)	51 (33.5%)	***0.0141***	29 (43.3%)	18 (24.7%)	***0.0197***	33 (57.9%)	26 (38.8%)	***0.0339***	12 (54.5%)	47 (53%)	*0.5214*	22 (51.2%)	59 (33.3%)	***0.0297***	7 (8.9%)	9 (6.5%)	*0.5258*	14 (58.3%)	65 (33.7%)	***0.0179***
	Low	45 (51.1%)	101 (66.5%)		38 (56.7%)	55 (75.3%)		24 (42.1%)	41 (61.2%)		10 (45.5%)	53 (47%)		21 (48.8%)	118 (66.7%)		72 (91.1%)	129 (93.5%)		10 (41.7%)	128 (66.3%)	

There was still an expected significant association between the expression of hypoxic, glycolytic and acid resistant phenotype markers (**Table 5**).

Table 5. Association between the hypoxia, glycolytic and acid resistant phenotype markers within the series of invasive breast carcinomas.

		HIF-1α			GLUT1			CAIX			MCT1			MCT4		
		Positive	Negative	p	Positive	Negative	p	Positive	Negative	p	Positive	Negative	p	Positive	Negative	p
GLUT1	Positive	38 (69.1%)	56 (34.6%)	***<0.0001***												
	Negative	17 (30.9%)	106 (65.4%)													
CAIX	Positive	24 (44.4%)	26 (16.1%)	***<0.0001***	52 (38.5%)	14 (7,9%)	***<0.0001***									
	Negative	30 (55.6%)	135 (83.9%)		83 (61,5%)	163 (92,1%)										
MCT1	Positive	27 (27%)	58 (28.7%)	*0.7554*	52 (39.7%)	26 (16.4%)	***<0.0001***	26 (40.6%)	52 (23.5%)	***0.0069***						
	Negative	73 (73%)	144 (71.3%)		79 (60.3%)	133 (83.6%)		38 (59.4%)	169 (76.5%)							
MCT4	Positive	26 (26.3%)	30 (14.4%)	***0.012***	38 (28.6%)	26 (15%)	***0.0039***	18 (27.7%)	45 (19.4%)	*0.1486*	29 (27.9%)	38 (12.8%)	***0.0004***			
	Negative	73 (73.7%)	178 (85.6%)		95 (71.4%)	147 (85%)		47 (72.3%)	187 (80.6%)		75 (72.1%)	258 (87.2%)				
CD147	Positive	7 (11.1%)	4 (5.6%)	*0.249*	12 (23.5%)	2 (3.6%)	***0.0025***	8 (38.1%)	7 (8.3%)	***0.0005***	19 (45.2%)	5 (2.9%)	***<0.0001***	5 (31.3%)	19 (9.6%)	***0.0083***
	Negative	56 (88.9%)	67 (94.4%)		39 (76.5%)	53 (96.4%)		13 (61.9%)	77 (91.7%)		23 (54.8%)	167 (97.1%)		11 (68.7%)	179 (90.4%)	

Interestingly, P-cadherin overexpression was also significantly associated with the expression of HIF-1α (*p<0.0001*), GLUT1 (*p<0.0001*), CAIX (*p<0.0001*), MCT1 (*p=0.0337*) and CD147 (*p<0.0001*) (**Table 6**); in contrast, no association was found with MCT4 expression *(p=0.553)*.

Table 6. Association between aberrant P-cadherin overexpression and the expression of proteins involved in hypoxic/glycolytic metabolism and acidic microenvironmental regulation, namely HIF-1α, GLUT1, CAIX, MCT1, MCT4 and CD147, in a series of invasive breast carcinomas.

		HIF-1α			GLUT1			CAIX			MCT1			MCT4			CD147		
		Positive	Negative	p	Positive	Negative	p	Positive	Negative	p	Positive	Negative	p	Positive	Negative	p	Positive	Negative	p
P-cadherin	Positive	48 (46.2%)	51 (24.4%)	***<0.0001***	60 (43.5%)	35 (18.8%)	***<0.0001***	38 (57.6%)	56 (22.7%)	***<0.0001***	43 (41%)	89 (29.7%)	***0.0337***	24 (34.8%)	109 (31.1%)	*0.5527*	18 (75%)	64 (33.3%)	***<0.0001***
	Negative	56 (53.8%)	158 (75.6%)		78 (56.5%)	151 (81.2%)		28 (42.4%)	191 (77.3%)		62 (59%)	211 (70.3%)		45 (65.2%)	241 (68.9%)		6 (25%)	128 (66.7%)	

With this data, we could demonstrate that breast carcinomas with positive expression to HIF-1α, GLUT1, CAIX, MCT1 and CD147 are significant associated to tumours showing a high percentage of cancer cells stained for the basal epithelial marker P-cadherin (**Figure 15**).

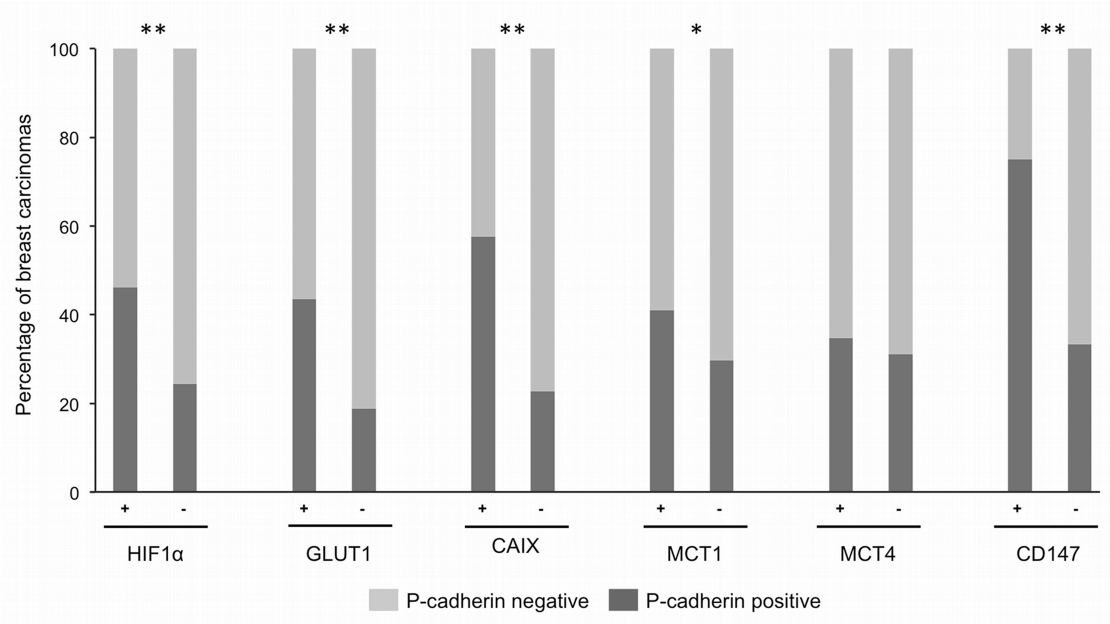


Figure 15. Aberrant P-cadherin expression in HIF-1α, GLUT1, CAIX, MCT1, MCT4 and CD147 expressing breast carcinomas. P-cadherin overexpression is significantly associated with the expression of HIF-1α (*p<0.0001*), GLUT1 (*p<0.0001*), CAIX (*p<0.0001*), MCT1 (*p=0.0337*) and CD147 (*p<0.0001*). No association was found with MCT4 expression *(p=0,553)*. **p<0.05*; ***p<0.0001*

II. P-CADHERIN MODULATION BY HYPOXIA AND ITS INVOLVEMENT IN GLYCOLYTIC AND ACID RESISTANCE PHENOTYPE IN BREAST CANCER

The evidences found concerning the association between P-cadherin expression and the development of a hypoxic/glycolytic and acid resistant phenotype in breast cancer cells, led us to further understand this putative link. We then decided to study the *in vitro* effect of hypoxia and HIF-1α in the expression of P-cadherin in breast cancer cells. We further evaluated the link between P-cadherin and the molecular machinery responsible for glycolytic and acid resistant phenotype, which is also known as playing a role in breast CSC survival and expansion. Due to the implications of P-cadherin expression in stem-like properties of breast cancer, we also evaluated the function of HIF-1α, GLUT1 and CAIX in the ability to survive to anchorage-independent conditions, as well as their association with P-cadherin related CSC markers in breast carcinomas.

IIa) P-cadherin expression is modulated by hypoxia in breast cancer cells

In order to evaluate the effect of hypoxia in P-cadherin expression of breast cancer cells, we cultured BT20 and SUM149 cells at atmosphere oxygen levels (Normoxia, 21% of oxygen) and at low oxygen levels (Hypoxia, 1% of oxygen). Using western blot, we were able to observe that hypoxia has a time dependent effect in P-cadherin expression of breast cancer cells (**Figure 16**). Short-time incubations in low oxygen tension, until approximately 24 hours, showed an increase of P-cadherin expression levels (**Figure 16**); however, after 48 hours in hypoxia, breast cancer cells start to exhibit a decrease in P-cadherin expression comparing with cells cultured in normoxia, being these results more pronounced after 5 days in these conditions. As a control of the experiment, we could observe an increase of hypoxia-inducible CAIX expression in hypoxic conditions compared with normoxia (**Figure 16**).

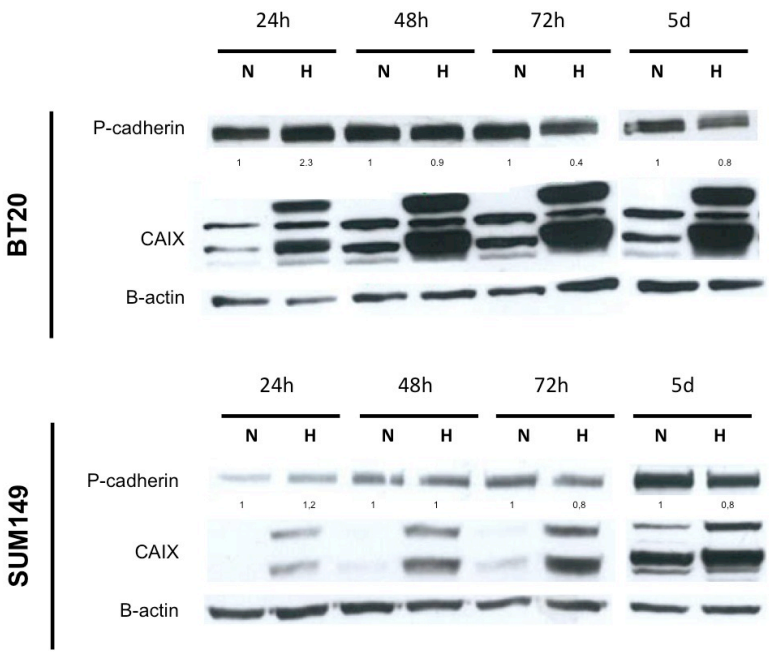


Figure 16. P-cadherin expression is modulated by hypoxia in a time dependent manner. Western blot shows an increase of P-cadherin expression in BT20 and SUM149 breast cancer cells when exposed to 1% of oxygen during 24hours. After 48hours of culture in hypoxia, P-cadherin expression is decreased in comparison to its expression in normal culture conditions. This effect is maintained until the 5th day in culture. CAIX expression increases in hypoxia comparing to normoxia conditions. (**N**: Normoxia, **H**: Hypoxia, **d**: days).

IIb) HIF-1α stimulation by $CoCl_2$ increases membrane P-cadherin expression in breast cancer cells

HIF-1α is the oxygen sensing subunit of HIF-1 heterodimer, involved in sensing and adaptation of cancer cells to low oxygen tensions. Due to the direct association found between P-cadherin and HIF-1α expression in invasive breast carcinomas, as well as to the effect of hypoxia in the modulation of P-cadherin expression in breast cancer cells, we decided to evaluate if HIF-1α was being responsible for the modulation of the expression of P-cadherin in breast cancer cells.

Treatment of SUM149 breast cancer cells with $CoCl_2$, a chemical stabilizer of HIF-1α, resulted, as expected, in an increased HIF-1α expression (**Figure17**). However, we could not observe any alterations in *CDH3* expression, either at mRNA or protein levels (**Figure17**), in comparison with the cells treated only with the vehicle.

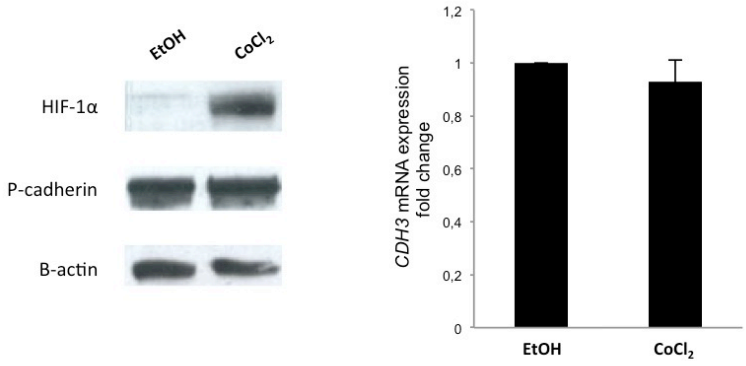


Figure 17. HIF-1α accumulation has no effect in *CDH3* expression either at the mRNA or at total protein level in SUM149 breast cancer cells. $CoCl_2$ leads to an increase of HIF-1α expression when compared with the cells treated with the vehicle (ethanol), whereas the levels of *CDH3* mRNA and total protein expression do not present any alterations (EtOH: ethanol).

Nevertheless, by FACS analysis, we could observe a statistically significant increase of membrane P-cadherin expression upon HIF-1α stabilization (*p=0.0246*; **Figure 18A** and **B**). We confirmed this result by confocal microscopy (**Figure 18C**), where we could notice that $CoCl_2$ treatment resulted in nuclear accumulation of HIF-1α, as well as in an increased expression of P-cadherin at the cell membrane, when compared with cells treated only with the vehicle (ethanol). Although not statistically significant (*p=0.0716*), we also observed a decrease in the cellular height after $CoCl_2$ treatment (**Figure 18D**), indicating a re-organization of the cytoskeleton after HIF-1α stabilization, which can be associated with the induction of P-cadherin expression.

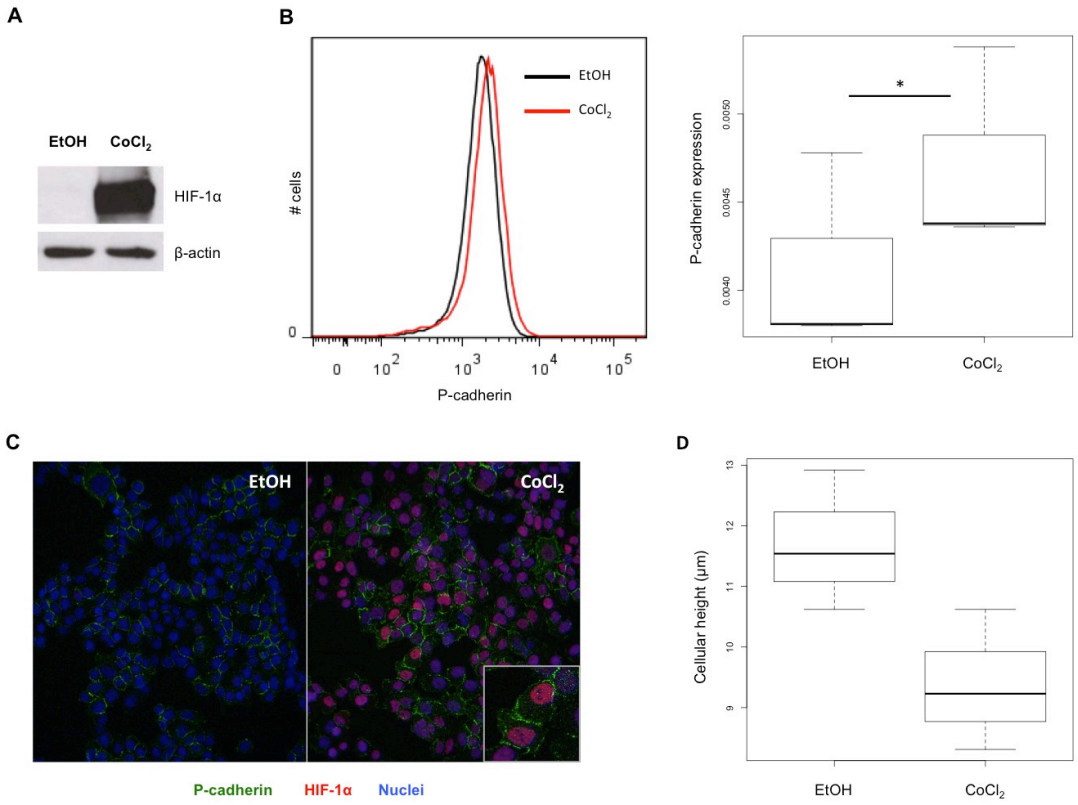


Figure 18. HIF-1α stimulation with $CoCl_2$ induces the expression of P-cadherin in the membrane of SUM149 breast cancer cells. HIF-1α stabilization and accumulation by $CoCl_2$ treatment was confirmed by western blot (A). Using FACS analysis, we observe a statistically significant increase (*p=0.0246*) in the expression of membrane P-cadherin in $CoCl_2$ treated cells when compared with the control cells treated with the vehicle (EtOH) (B). Immunofluorescence of $CoCl_2$-treated cells shows nuclear HIF-1α expression (red) and an increase of membrane P-cadherin expression (green) compared with vehicle treated cells (C). Z-stack measurements reveal a decrease in the height of $CoCl_2$-treated cells comparing with the control cells (*p=0,0716)* (D).

IIc) P-cadherin expression interferes with *GLUT1* and *CAIX* mRNA levels in breast cancer cells

To further explore if there was a crosstalk between P-cadherin expression and the machinery involved in the glycolytic and acid resistance phenotype in breast cancer cells, we decided to silence *CDH3* transcripts by siRNA-mediated knockdown in TN/basal-like P-cadherin overexpressing breast cancer cell models (BT20 and SUM149). By real-time PCR, we could observe that *CDH3* silencing leads to a statistically significant downregulation of *GLUT1* and *CAIX* mRNA in BT20 breast

cancer cells (p<0.05) (**Figure 19A**). Although not statistically significant, we could also find a tendency to a decrease in *GLUT1* and *CAIX* mRNA levels in SUM149 breast cancer cells (**Figure 19D**). No significant alterations were found in the mRNA expression of *HIF-1α, MCT1* and *CD147* upon the silencing of *CDH3* in both cell lines (**Figure 19A** and **D**). Interestingly, when *GLUT1* (**Figure 19B** and **E**) and *CAIX* (**Figure 19C** and **F**) were silenced in both cell lines, there were no significant alterations in *CDH3* mRNA levels.

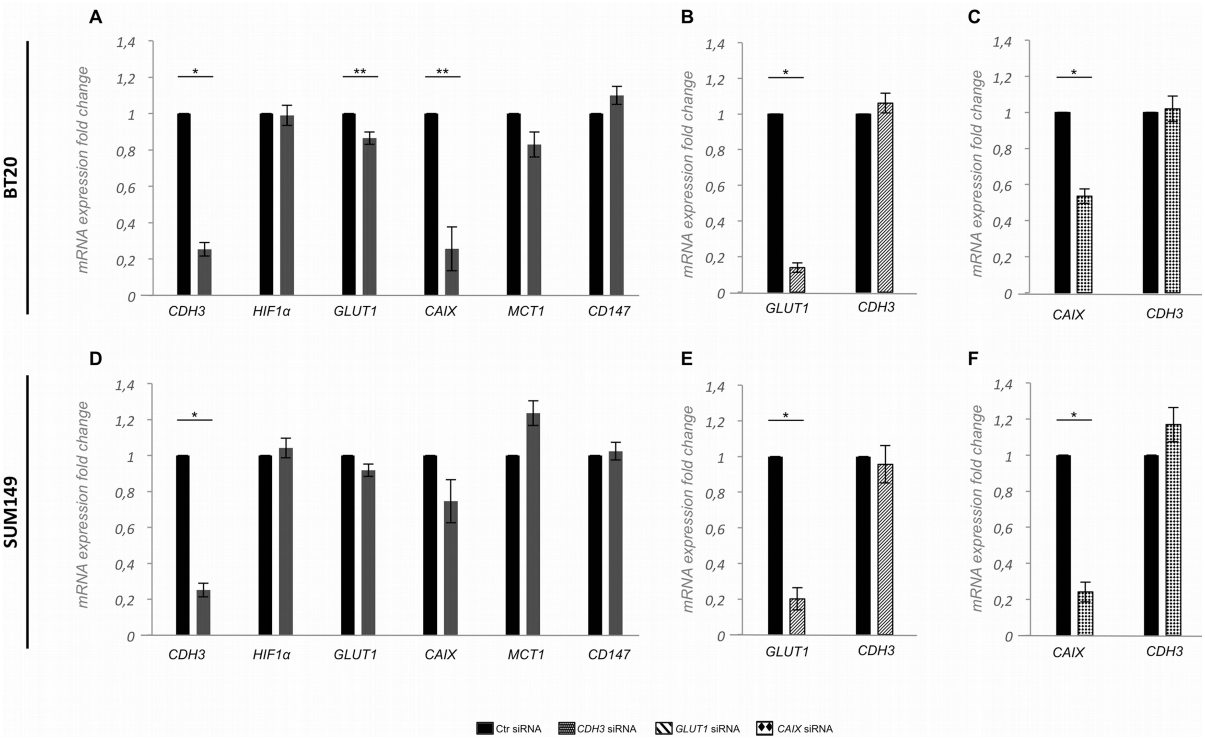


Figure 19. P-cadherin expression affects *GLUT1* and *CAIX* mRNA levels in breast cancer cells. mRNA expression measured by qRT-PCR of *CDH3*, *HIF-1α*, *GLUT1, CAIX, MCT1 and CD147* when inducing siRNA-mediated knockdown of *CDH3*, *GLUT1* and *CAIX* in BT20 and SUM149 breast cancer cell lines. Upon *CDH3* silencing in BT20 cells (A), there is significant decrease of the mRNA expression of *GLUT1* and *CAIX* in BT20 (A) and a tendency, although not statistically significant, of decrease in SUM149 cells (D). No differences are observed in the mRNA expression of *HIF-1α, MCT1 and CD147* in both cell lines. On the other hand, there are no alterations in *CDH3* mRNA expression in BT20 (B and C) and in SUM149 (E and F) breast cancer cell lines, when we silence *GLUT1 (B and E)* and *CAIX (C and F)*. **p<0.05*; ***p<0.0001*.

IId) P-cadherin is co-expressed with GLUT1 and CAIX in basal-like breast cancer cell lines

The above results led us to go further on the relationship between P-cadherin and GLUT1 and CAIX, since we observed that the expression of these both molecules was being responsive to P-cadherin in breast cancer cells. Thus, we decided to study if there was an enrichment of P-cadherin expression in GLUT1 and/or CAIX positive populations. Interestingly, we found that P-cadherin is co-expressed with GLUT1 and CAIX in triple negative BLBC cell lines (**Figure 20**).

We observed that SUM149 cells presenting the highest expression of P-cadherin (20% high P-cad) were the ones also presenting the highest expression of GLUT1 and CAIX, while the ones showing the lowest expression of P-cadherin (20% low P-cad) demonstrated the lowest levels of GLUT1 and CAIX (**Figure 20A**). This result was confirmed when we sorted and separated the 20% high and low P-cadherin cell populations and analysed the expression of GLUT1 and CAIX by western blot (**Figure 20B**). Furthermore, when we selected the population of cells by their GLUT1 expression, the 20% high/low GLUT1 cells also presented the highest and lowest levels of P-cadherin expression, respectively (**Figure 20C**). Still, the cells selected by CAIX expression also presented the same tendency concerning P-cadherin expression (**Figure 20D**). Similar results were obtained in BT20 breast cancer cells.

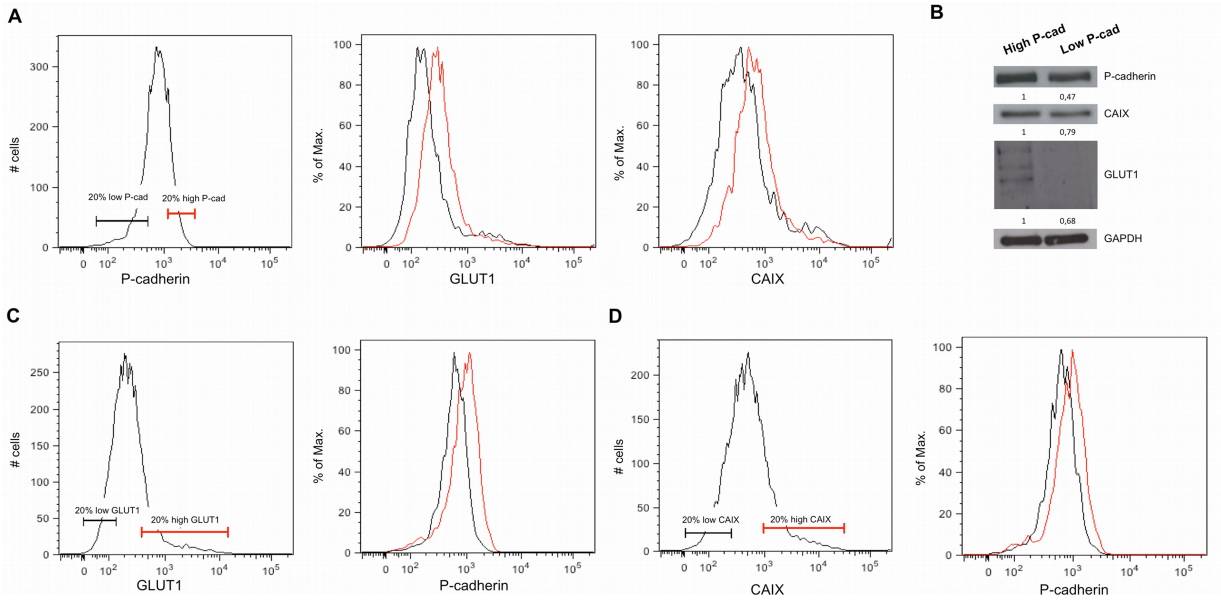


Figure 20. P-cadherin is co-expressed with GLUT1 and CAIX in triple negative and basal-like SUM149 breast cancer cell lines. By flow cytometry analysis, we observe that the 20% of cells with the highest and lowest P-cadherin expression presents highest and lowest, respectively, expression of GLUT1 and CAIX (A). When cells were sorted by P-cadherin expression, lysed and analysed in SDS-PAGE, the same result was observed concerning the high and low expression of GLUT1 and CAIX (B). On the other hand, 20% of cells with the highest and lowest levels of GLUT1 and CAIX (C and D, respectively) expression also present highest and lowest P-cadherin expression.

IIe) Hypoxic, glycolytic and acidosis biomarkers expression affects MFE in basal-like breast cancer cells and is associated with P-cadherin-related CSC markers in breast carcinomas

Since it has been already described that HIF-1α, GLUT1 and CAIX are required for CSC survival and tumour aggressiveness, and that we have recently shown that P-cadherin is also involved in the maintenance of stem-like properties of breast CSCs, we decided to evaluate the effect of the inhibition of all these molecules, alone or in combination, on the mammosphere forming efficiency (MFE%) of basal-like SUM149 model (**Figure 21**).

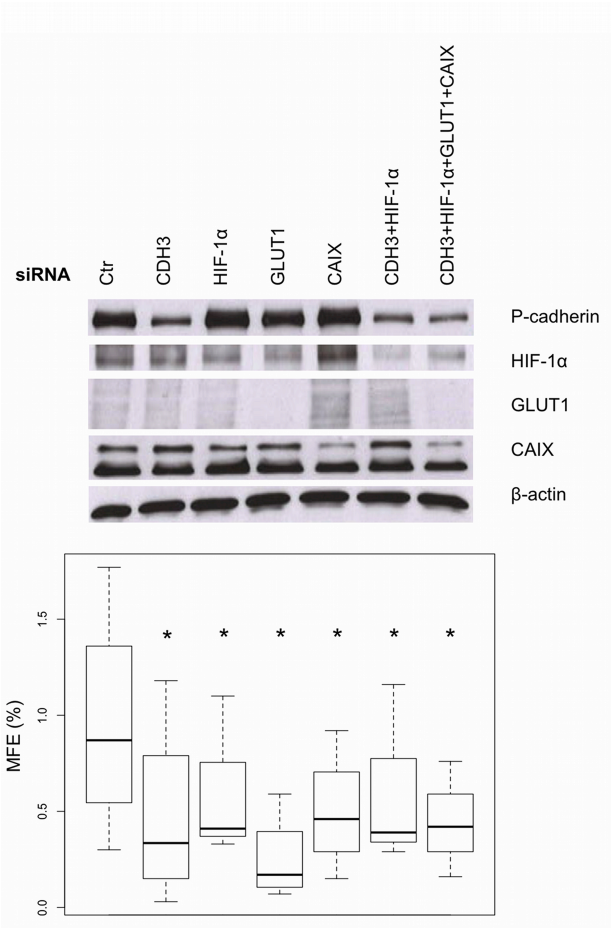


Figure 21. MFE (%) decreases when inducing siRNA-mediated silencing of *CDH3*, *HIF-1α, GLUT1* and *CAIX* in SUM149 breast cancer cells. A statistically significant decrease in MFE (%) is observed when we silence *CDH3 (p=0.0153), HIF-1α (p=0.0156), GLUT1 (p=0.000284)* and *CAIX (p=0.000902).* The simultaneous silencing of *CDH3* and *HIF-1α* also revealed a significant decrease of MFE (%) of the target cells (*p=0.0367*), although not cumulative. Still, the silencing of the expression of all transcripts tested, also leaded to a non-cumulative inhibition of the MFE compared to the control cells (*p=0.0152*). Protein levels of P-cadherin, HIF-1α, GLUT1 and CAIX were confirmed by western blot after specific siRNA silencing. **p<0.05*

As expected, silencing the expression of *CDH3,* as well as of *HIF-1α, GLUT1* and *CAIX,* showed a significant decrease of the ability to form mammospheres when compared with the cells transfected with the control siRNA (*p=0.0153*, *p=0.0156, p=0.000284* and *p=0.000902,* respectively). Moreover, when we simultaneously silenced *CDH3* and *HIF-1α,* we also observed a decrease of MFE of the target cells (*p=0.0367*), although not cumulative. If, in addition to *CDH3* and *HIF-1α,* we silence *GLUT1* and *CAIX* (siRNA *CDH3+HIF-1α+GLUT1+CAIX),* there is still a non-cumulative decrease effect in MFE (%) (*p=0.0152*).

Based on the above presented results, we also evaluated the association of HIF-1α, GLUT1 and CAIX with the CSC markers, CD44 and CD49f (**Table 7**), already described to be enriched in P-cadherin overexpressing tumours, using a previously

characterized and independent series of 466 primary invasive breast carcinomas [174, 306]. Accordingly, we were able to validate the associations above described of P-cadherin expression with HIF-1α and GLUT1 (*p=0.009 and p<0.001*, respectively); however, no association was found between P-cadherin and CAIX expression (*p=0.819)* (**Table 7**).

As previously reported, P-cadherin expressing tumours were significantly enriched in the expression of CD44 (*p=0.003*) and CD49f (*p=0.001*). Interestingly, we were able to find that GLUT1 was significantly associated with the expression of CD44 and CD49f (*p=0.002 and p=0.001, respectively)*. Moreover, CAIX expression was found to be associated with CD44 expression (*p=0.017);* no association was found between CAIX and CD49f expression *(p=0.877)*. Still, no significant associations were found between HIF-1α and CD44 and CD49f (*p=0.168* and *p=0.062*, respectively) (**Table 7**).

Table 7. Association between P-cadherin-related CSC markers and hypoxia, glycolytic and acidosis markers in primary invasive breast cancer.

		CD44			CD49f		
		Positive	Negative	p	Positive	Negative	p
P-cadherin	Positive	72 (30.4%)	42 (18.6%)	**0.003**	29 (59.2%)	77 (20.4%)	**0.001**
	Negative	165 (69.6%)	184 (81.4%)		20 (40.8%)	301 (79.6%)	
HIF-1α	Positive	99 (46.9%)	83 (40.1%)	0.168	27 (57.4%)	154 (42.8%)	0.062
	Negative	112 (53.1%)	124 (59.9%)		20 (42.6%)	206 57.2%)	
GLUT1	Positive	50 (23.3%)	24 (11.8%)	**0.002**	18 (36.7%)	55 (15.1%)	**0.001**
	Negative	165 (76.7%)	180 (88.2%)		31 (63.3%)	310 (84.9%)	
CAIX	Positive	135 (64.6%)	111 (52.9%)	**0.017**	29 (60.4%)	211 (58.4%)	0.877
	Negative	74 (35.4%)	99 (47.1%)		19 (39.6%)	150 (41.6%)	

III. P-CADHERIN'S EFFECT IN BREAST CANCER CELL'S METABOLISM

The results presented before suggests that P-cadherin overexpressing breast cancer cells are most likely to exhibit increased glycolysis and to survive to metabolic driven pH alterations. In fact, it has been already shown that breast CSC present decreased OXPHOS, increased glycolytic metabolism and contain lower levels of ROS, providing them a protection against oxidative stress. Taken into account that P-cadherin is a survival factor in breast cancer cells, that it confers resistance to x-ray induced cell death, and also that it mediates stem cell properties in BLBC cells, we decided to further investigate the effect of P-cadherin in breast cancer cell metabolism, as well as in mitochondrial functional characteristics, such as ATP production, ROS levels and their scavenging systems and mitochondrial membrane potential.

IIIa) P-cadherin expression regulates metabolic activity and ATP content of basal-like breast cancer cells

In order to functionally evaluate the role of P-cadherin expression in cellular bioenergetics, we used Seahorse technology and analysed the oxidative phosphorylation, measured by oxygen consumption rate (OCR), as well as glycolysis, measured by ECAR.

Initially, we evaluated the metabolic behaviour of SUM149 and MDA-MB-468 BLBC cells. Basal OCR and ECAR was measured over time followed by mitochondrial function assays, where the effect of well characterized inhibitors of electron transport chain, such as oligomycin A and FCCP, was evaluated. **Figure 22** shows the metabolic profile of SUM149 and MDA-MB-468 cells.

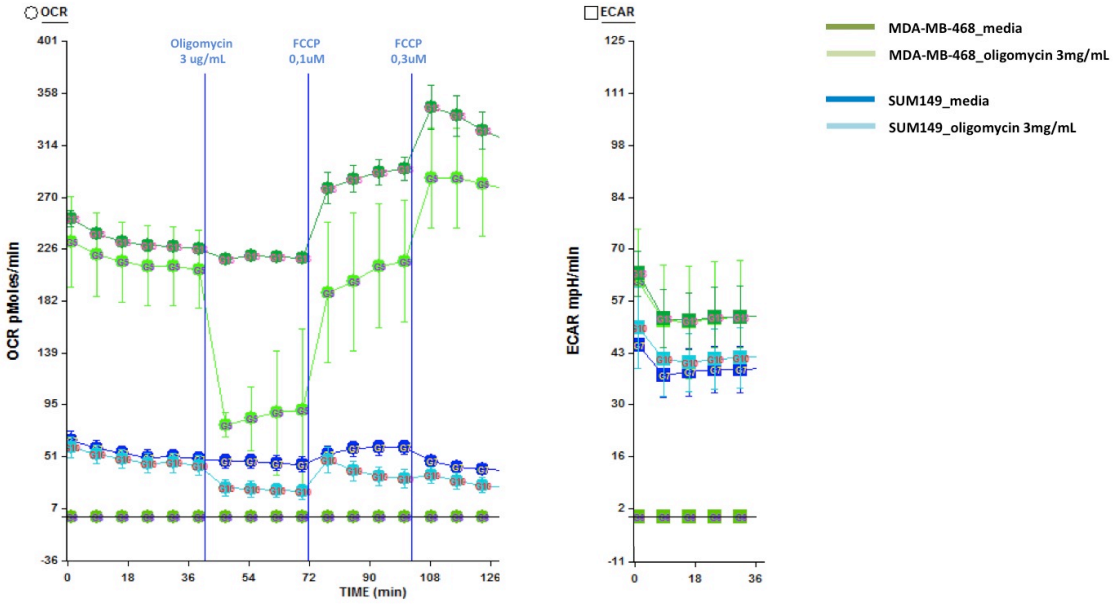


Figure 22. Bioenergetic profile of triple negative BLBC cells. Real time analysis of OCR and ECAR of SUM149 and MDA-MB-468 breast cancer cells at basal levels and OCR rates upon the injection of 3μg/mL of oligomycin and 0,1 and 0,3μM of FCCP.

SUM149 breast cancer cells presented a basal OCR rate of about 5-fold change lower than MDA-MB-468 cells, while ECAR was only slightly lower. Furthermore, the injection of 3ug/mL of oligomycin A showed a drastic effect in the OCR of MDA-MB-468, suggesting that these cells have a much higher ATP linked respiration than SUM149 (**Figure 22**). This profile also revealed that the maximum OCR (upon proton ionophore FCCP injection) is higher in MDA-MB-468 cells than in SUM149. This data indicates that SUM149 are more glycolytic than MDA-MB-468 cells meaning that these cells preferentially use glycolysis instead of mitochondrial oxidative phosphorylation to meet their energy demands, being a good model to evaluate the putative glycolytic promoter role of P-cadherin in breast cancer cells.

We have previously demonstrated that P-cadherin acts as a survival factor in BT20 breast cancer cells [296]. In this work, we were able to confirm the survival role of P-cadherin in SUM149 breast cancer cells. Apoptosis was increased upon *CDH3* siRNA-mediated downregulation in SUM149 cells (**Figure 23**).

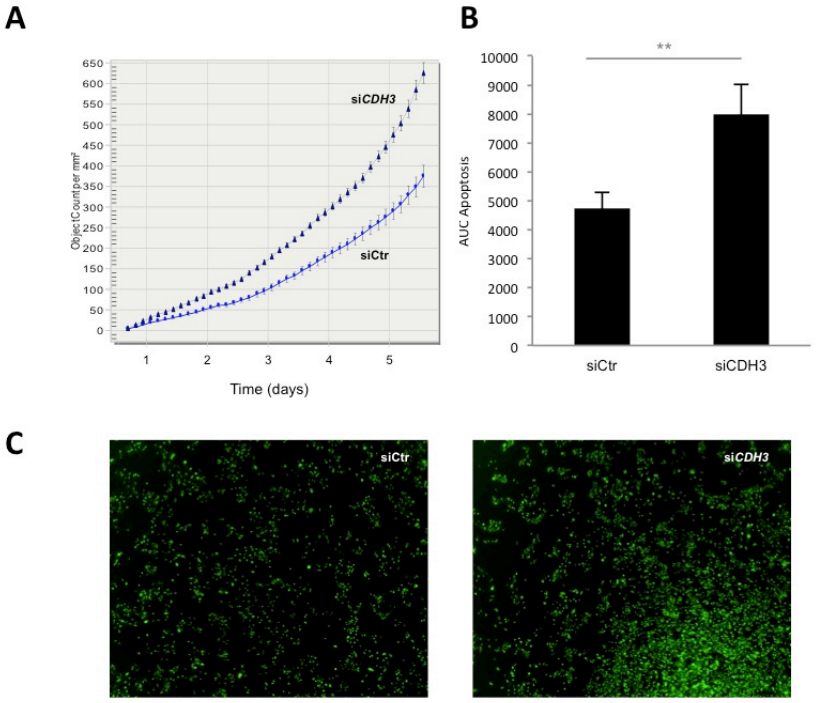


Figure 23. P-cadherin is a survival factor in BLBC cells. SUM149 breast cancer cells with *CDH3* silencing present a higher number of apoptotic cells comparing with the control cells (A). Quantification of the AUC (area under curve) of apoptotic graphs shows a statistically significant increase of cell death in *CDH3* silenced cells (B). Representative image of apoptotic cells (green) in control and *CDH3* siRNA transfected SUM149 breast cancer cells (C). *p<0,05 **p<0,001

We then analysed the basal OCR and ECAR rates in highly glycolytic SUM149 with *CDH3* downregulation. Interestingly, we observed that P-cadherin silencing (**Figure 24A**) induces an increase of the basal OCR/ECAR ratio comparing to control cells (siRNA Ctr) (**Figure 24B**), indicating a metabolic shift towards oxidative phosphorylation.

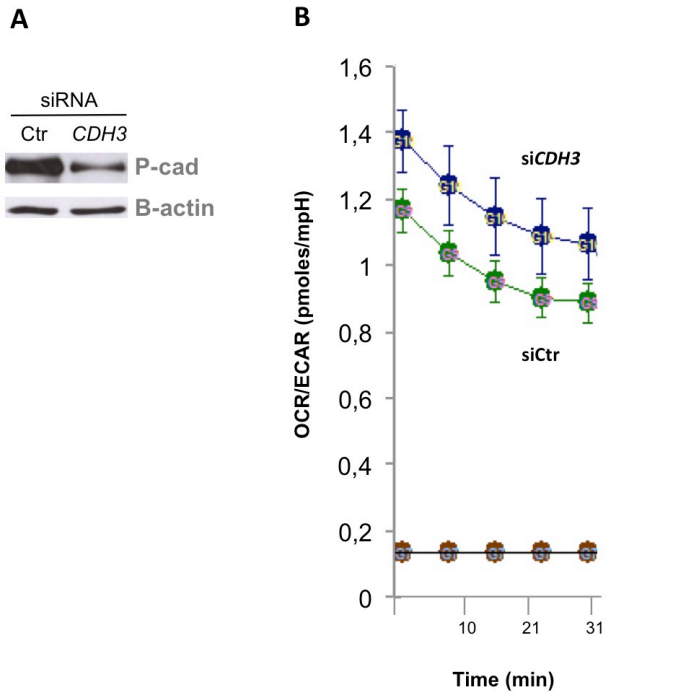
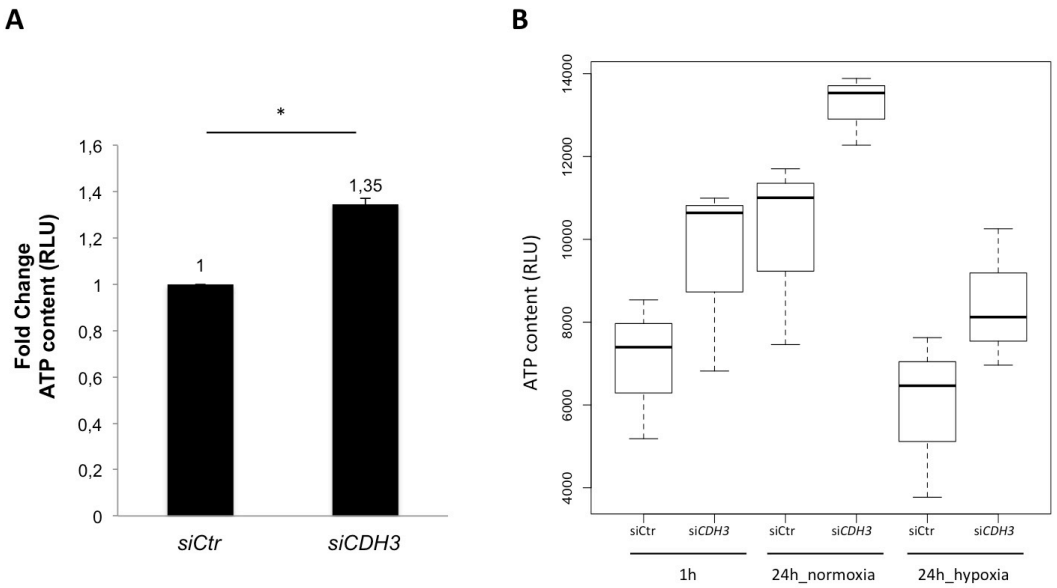


Figure 24. P-cadherin inhibition increases OXPHOS in SUM149 breast cancer cells. Western blot showing P-cadherin knockdown in siRNA-mediated *CDH3* silenced SUM149 comparing to the control cells (A). P-cadherin knockdown induces an increase in OCR/ECAR ratio comparing to the control cells (B).

Since OXPHOS levels were increasing upon P-cadherin knockdown, we then decided to evaluate the ATP content in SUM149 breast cancer cells. Accordingly, we observed a statistically significant increase (*p=0.0174)* of approximately 35% in the ATP content of *CDH3* silenced breast cancer cells compared with the control (**Figure 25A**).

ATP levels increased after 24 hours of seeding the cells, when compared to measurements at 1 hour after seeding, as expected by cell proliferation and increased metabolic activity. Moreover, hypoxia cultured cells decreased their ATP content comparing to the cells exposed to atmospheric oxygen levels for the same 24 hours, which was also expected by the metabolic shift towards glycolysis observed in hypoxia. Interestingly, the increase of ATP content in *CDH3* silenced breast cancer



cells was observed 1 hour after seeding, as well as 24 hours later, in normoxia and in hypoxia conditions (**Figure 25B**).

Figure 25. P-cadherin expression modulates ATP content in SUM149 breast cancer cells. ATP content is increased in SUM149 cells with *CDH3* downregulation in comparison to control cells (*p=0.0174*) (A). After 24hours in culture, SUM149 cells presented higher levels of ATP in relation to the ATP levels after one hour of seeding the cells; ATP levels are lower when SUM149 cells were culture during 24 hours in hypoxia comparing to normoxia (B). **p<0.05*.

Using glycolysis-targeting compounds, we were able to measure the ATP derived from glycolysis, as a measure of percentage of glycolysis. Surprisingly, there were no differences between SUM149 cells with *CDH3* downregulation in comparison with the control cells. Moreover, ATP derived from glycolysis was similar in SUM149 cultured in normoxic and in hypoxic environments.

These results imply that P-cadherin expression could be interfering with the metabolic behaviour of breast cancer cells at the oxidative phosphorylation, rather than at the glycolysis level. In an attempt to understand if the effect of P-cadherin could be through the interference with OXPHOS regulators, we then focused on the expression of phosphorylated PDH (pyruvate dehydrogenase) and PDK2 (pyruvate

dehydrogenase kinase 2), which activity can modulate oxidative metabolism. However, we were not able to see any differences concerning p-PDH and PDK2 expression (**Figure 26**).

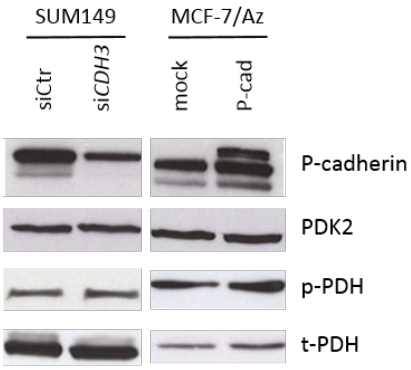


Figure 26. P-cadherin expression does not interfere with the expression of OXPHOS modulators. Western blot shows no differences in the expression of PDK2 as well as in phosphorylated and total levels of PDH.

IIIb) P-cadherin expression induces alterations in ROS levels of breast cancer cells through the modification of detoxifying enzymes

Since ROS are able to induce cell death, either by apoptosis or anoikis, and mitochondrial respiration is the major source of ROS, we decided to evaluate the production of ROS upon P-cadherin modulation in breast cancer cells (**Figure 27**). We were able to observe that *CDH3* silencing leads to a statistically significant increase ($p<0.05$) of ROS levels in MDA-MB-468 cells when compared to control cells (**Figure 27A**). Although not statistically significant, we could also find a tendency to an increase in ROS in BT20 and SUM149 cells, when a downregulation of P-cadherin expression was induced (**Figure 27B** and **C**, respectively). Furthermore, ROS levels decreased in MCF-7/Az.P-cadherin cells, where P-cadherin is overexpressed, comparing to MCF-7/Az.mock cells ($p<0.05$) (**Figure 27D**).

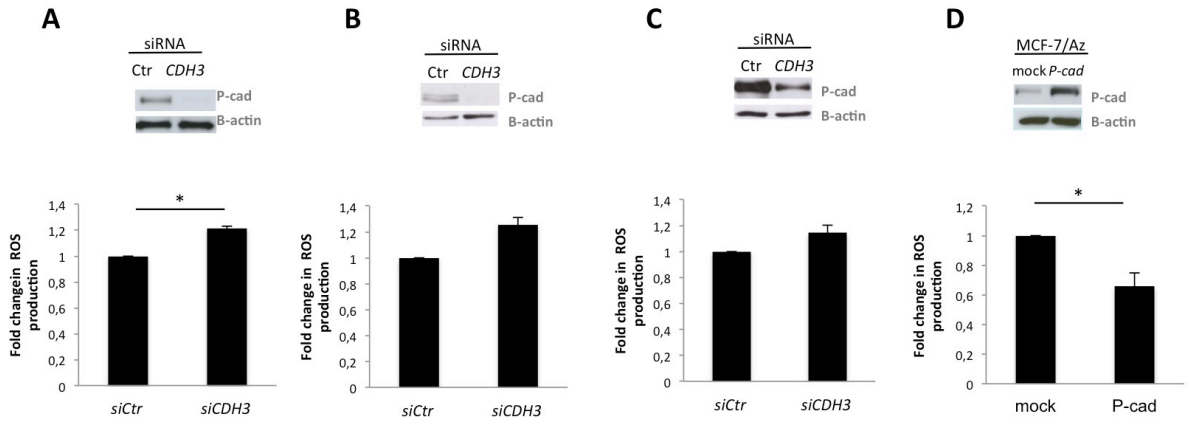


Figure 27. P-cadherin expression regulates ROS production in breast cancer cells. Compared with the control cells (si*Ctr*), inhibition of P-cadherin expression leads to a statistically significant increase (*p=0.0123*) of ROS production in MDA-MB-468 (A) as well as in SUM149 (B) and BT20 (C), although not statistically significant (*p=0.14*0 and *p=0.0716*, respectively). MCF-7/Az cells with overexpression of P-cadherin shows lower levels of ROS comparing to mock cells (D, *p=0,0434*). Western blot confirmed the inhibition and overexpression of P-cadherin in all the models presented. **p<0.05*.

Aiming to address if the variations in ROS levels could be associated with alterations in scavenging systems, we evaluated the expression and activity of detoxifying enzymes, such as Cu/Zn-SOD (SOD1) and Mn-SOD (SOD2) (**Figure 28**). *CDH3* silencing in SUM149 breast cancer cells led to a statistically significant decrease *(p=0.000299)* of SOD1 expression (**Figure 28A**), as well as to a significant decrease *(p=0.0228)* in SOD2 activity (**Figure 28D**), which was consistent with the increased ROS levels in these cells when compared to control cells. Furthermore, MCF-7/Az cells with overexpression of P-cadherin, where ROS levels were found to be downregulated, showed an increased expression of SOD1 *(p=0.0399)* and, although not statistically significant, we could also observe a tendency for an increase in SOD2 activity in cells with overexpression of P-cadherin *(p=0.202)*. We did not observe any significant alterations in SOD 1 activity as well as SOD2 expression in breast cancer cells with *CDH3* altered expression (**Figure 28B** and **C**, respectively).

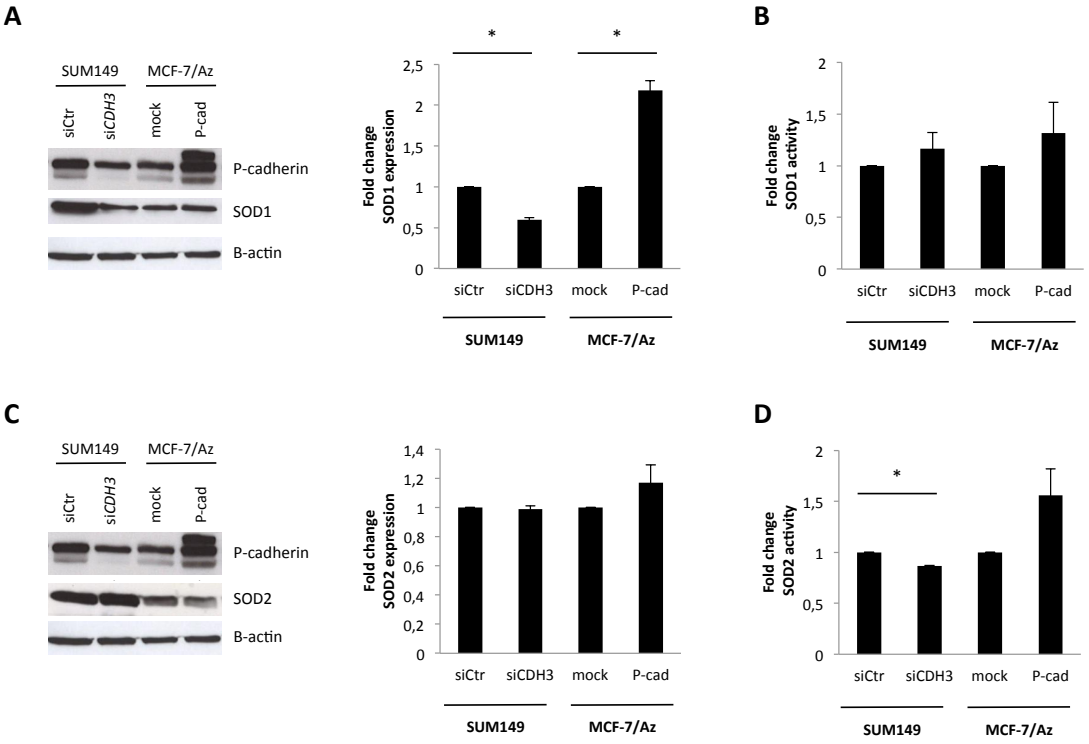


Figure 28. P-cadherin expression modulates antioxidant systems in breast cancer cells. Inhibition of P-cadherin expression in SUM149 cells leads to a statistically significant decrease in SOD1 expression, while P-cadherin overexpressing MCF-7/Az cells presented higher levels of SOD1 expression, comparing to the respective control cells. No alterations in SOD1 activity were observed upon *CDH3* deregulation (B). On the other hand, SOD2 expression did not show any significant alterations in breast cancer cells when P-cadherin expression was modulated (C); however, SOD2 activity decreased significantly when inducing *CDH3* downregulation and, although not statistically significant, MCF-7/Az cells with overexpression of P-cadherin shows higher SOD2 activity comparing to mock cells (D).

IIIc) Effect of P-cadherin in mitochondrial function

Given the alterations in the production of ROS, as well as the metabolic shift and ATP production induced by P-cadherin expression in breast cancer cells, we decided to evaluate its effect in the mitochondrial membrane potential (**Figure 29**). We were not able to find any significant alteration in the fluorescence intensity of Mitotracker CMXRos when we silenced P-cadherin expression in SUM149 breast cancer cells (**Figure 29A**). Still, low P-cadherin expressing or P-cadherin-enriched cell populations did not present significant differences in mitochondrial membrane potential (**Figure**

29B). However, further studies are still needed to comprehend the overall role of P-cadherin in mitochondrial functional characteristics.

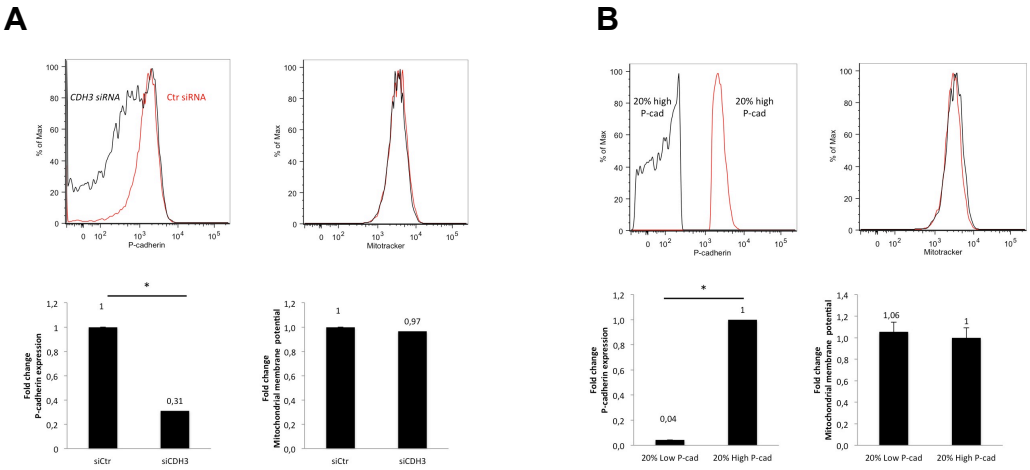


Figure 29. P-cadherin expression has no effect in mitochondrial membrane potential. Mitotracker CMXRos fluorescence is not altered in SUM149 breast cancer cells when inducing *CDH3* downregulation (A). Cell population with the higher and lower P-cadherin expression did not present alterations in mitochondrial membrane potential measured by Mitotracker CMXRos fluorescence in FACS analysis (B).

CHAPTER V

Discussion

The results presented in this work provide novel insights about the role of P-cadherin expression in microenvironment adaptation by breast cancer cells as well as the possible implications of its expression in breast cancer progression and development of metastasis.

P-CADHERIN OVEREXPRESSION IS SIGNIFICANTLY ASSOCIATED WITH THE EXPRESSION OF HYPOXIC, GLYCOLYTIC AND ACIDOSIS MARKERS IN PRIMARY INVASIVE BREAST CARCINOMAS.

In the last years, there is an increased recognition that microenvironmental stress that solid tumours are exposed to, such as hypoxia, lactic acidosis and glucose deprivation, play an important role in breast carcinogenesis [18, 320]. Interestingly, the immunohistochemical expression of HIF-1α, GLUT1 and CAIX in a histological model of breast cancer progression, including normal mammary gland, ductal hyperplasia (DH), atypical ductal hyperplasia (ADH), *in situ* and invasive ductal breast carcinomas, Chen *et al.* proposed that this phenotype is a powerful adaptive advantage associated to an aggressive phenotype in breast carcinomas [89].

In this work, we demonstrate, for the first time, that aberrant P-cadherin expression, a well described poor prognostic factor in breast cancer and an inducer of aggressive behaviour of breast cancer cells *in vitro*, is associated with the hypoxic/glycolytic and acid resistant phenotype in breast carcinomas, evaluated by a panel of markers including HIF-1α, CAIX, GLUT1, MCT1 and CD147.

Although it is well established the association between both HIF-1α and P-cadherin with several features of aggressive breast tumour behaviour, our results demonstrated for the first time a significant association between P-cadherin and HIF-1α expression in breast cancer. It is widely known that, similarly to P-cadherin, HIF-1α expression in breast cancer is also associated to worse prognosis, short patient's survival, high proliferation and poor tumour differentiation [43, 47].

Unexpectedly, there was no statistically significant association between HIF-1α and ER in this breast cancer series. However, we could find an enrichment of HIF-1α positivity in ER negative breast tumours. We also found that HIF-1α expression is predominant in highly proliferative and high-grade tumours, but no correlation was found with age of the patients' diagnosis, size of the tumour, lymph node invasion, molecular subtype, nor molecular biomarkers of breast cancer such as ER, PgR and HER2. These results are in accordance with Tan *et al.*, since they found that HIF-1α expression was not associated with age, size, ER and HER2 *[91]*. Moreover, we also found an association of HIF-1α expression with its downstream targets, such as CAIX and GLUT1, which is in accordance with previous reports [89, 321].
Although there are several reports in the literature about immunohistochemical HIF-1α expression in breast cancer, there is a high degree of contradictory observations among these studies concerning associations with hormone receptors, HER2, lymph nodes and histological grade [54, 322]. These differences may be due to the use of different classification criteria for HIF-1α, or to the analyses of specific tumours areas, or even to the use of tissue microarrays (TMA), especially for a marker that presents so much intratumoural heterogeneity as HIF-1α [323].

Several studies have been reporting that BLBC present a stronger response to hypoxia than tumours with luminal characteristics. This group of tumours, where P-cadherin is frequently overexpressed, is particularly relevant in the clinical setting, since they are associated with aggressive tumour behaviour and shorter overall survival when compared with other molecular subtypes of breast tumours. Phenotypically, these tumours are characterized by the lack of the expression of hormone receptors (ER and PgR) and HER2; and histologically, they are poorly differentiated carcinomas, with high nuclear and histological grade and frequently show medullary and metaplastic features [284-288]. A distinct pattern of metastasis to brain and lungs, known to be associated with poor prognosis, and less significant involvement of axillary lymph nodes, has also been described in BLBC [170, 288, 289]. Interestingly, these tumours also present a differential expression of proteins induced by hypoxia and the development of a glycolytic/acid resistant phenotype [71,

91, 99], which confers a selective advantage to cancer cells to grow and escape cell death under these adverse conditions. Accordingly, our results also showed an association between P-cadherin expression and HIF-1α downstream targets, such as GLUT1, CAIX, MCT1 and CD147. These proteins are important stress response mediators of cancer cell survival in stressful microenvironments, being associated with poor patient prognosis in breast cancer [71, 91, 99].

In fact, it is expected that triple negative and BLBC show a high metabolic activity [324-327]. Interestingly, Kim *et al.* evaluated the metabolic phenotype among the different molecular subtypes of triple negative breast carcinomas, based on the immunohistochemical expression profiles of GLUT1 and CAIX, representing a Warburg, Reverse Warburg, mix and null phenotype, taken into account their expression in tumour and stromal cells. In consistence with our results, they observed that triple-negative breast cancer (TNBC) presented a highly glycolytic activity in tumour cells and, consequently, a Warburg molecular phenotype. However, they did not found any significant association between the metabolic phenotype and the molecular subtypes of TNBC [328].
It has also been reported by several other authors that the expression of GLUT1, CAIX, MCT1 and CD147 is associated with aggressive clinico-pathological characteristics in primary invasive breast carcinomas such as high proliferation rates, high histological grade and poor patient's survival and are also known to be differentially expressed in basal-like breast carcinomas, in addition to their association with molecular characteristics of BLBC, such as absence of hormone receptors and expression of key basal markers such as CK5, EGFR, CK14 and Vimentin [71, 91, 99, 326, 329].

Moreover, we did not find association between MCT4 and P-cadherin expression in breast carcinomas when evaluating its expression in tumour cells. In the literature, there are contrasting results concerning MCT4 expression in breast cancer. For example, Pinheiro *et al.* did not find any significant association between MCT4 and clinicopathological data [99]. However, prognostic value was attributed to MCT4 when

expressed in the stromal compartment of the tumour [328]. Kim *et al*. found that stromal MCT4 absence was associated with short overall survival of breast cancer patients [328]. In contrast, stromal MCT4 expression was previously described as a predictive factor of decreased overall survival in TNBC [330]. These conflicting results can be explained by the use of different methodologies, namely in the classification method, as well as in the antibodies used to access the expression of MCT4.

As stated above, in order to avoid acidosis-induced cell death caused by increased glycolytic rates, membrane transporters, such as CAIX and MCTs, are upregulated in order to maintain pH homeostasis inside the cells. Consequently, this cellular behaviour induces extracellular acidification, which is known to facilitate *in vitro* cancer cell invasion and *in vivo* metastization, through the acidic degradation of the extracellular matrix [82]. CD147/EMMPRIN, a molecular chaperone of MCT1 and MCT4, is known to induce MMP production [102], to promote tumour growth, inhibit cell apoptosis and enhance cell migration under hypoxic conditions [331]. Using *in vivo* models, it was also demonstrated that EMMPRIN-induced expression in breast cancer cells resulted in enhanced tumour growth in nude mice, associated to MMP2 expression [104]. Interestingly, the high significant association found between P-cadherin and CD147 is in accordance not only with the above-described reports, but also with our previous data, showing that P-cadherin induces an invasive behaviour in breast cancer cells by a mechanism involving the activation of MMP1 and MMP2 [295].

In addition to its differential expression in BLBC, P-cadherin is also enriched in HER2 overexpressing breast tumours [228, 230, 332]. Interestingly, breast tumours with simultaneous expression of HIF-1α and HER2 present worse survival, indicating that HER2-mediated tumour aggressiveness in breast cancer can be partially due to HIF-1α activation [333].

Taken together, the association between markers of hypoxic, glycolytic and acid-resistance phenotype and P-cadherin expression in primary invasive breast carcinomas, is consistent with the relevant data concerning their expression in breast carcinomas.

P-CADHERIN EXPRESSION IS MODULATED BY HYPOXIA AND IS CONNECTED TO THE MACHINERY INVOLVED IN GLYCOLYTIC AND ACID RESISTANCE PHENOTYPE IN BREAST CANCER CELLS

Based on the concept that P-cadherin, hypoxia and metabolic alterations are associated with poor prognosis in breast cancer, as well as on the associations found between P-cadherin and biomarkers of this phenotype in breast carcinomas, we decided to study if P-cadherin expression has a role in the adaptation of breast cancer cells to hypoxia and to evaluate its involvement in the regulation of the machinery responsible for the acquisition of this adapted glycolytic and acid resistant phenotype.

Hypoxia has been recognized as being determinant for clinical outcomes in human cancers. In fact, several studies have been validating gene signatures associated with hypoxia as a prognostic factor in several types of cancer, including breast cancer. For instance, Winter *et al*. obtained a hypoxia metagene of head and neck cancer (HNSCC), which was further found to have a poor prognosis relevance in independent data sets, including in breast cancer [41]. Moreover, Chi *et al.* showed that human tumours, including breast carcinomas, could be stratified according to the presence and amplitude of a hypoxia response, using a hypoxia-response signature derived from mammary epithelial cells [40]. These authors showed that breast tumours with a strong gene expression signature of hypoxia response presented a significantly worse prognosis, correlated with breast cancer progression and metastasis. They still demonstrated that the prognostic information of the hypoxia signature was independent of other previously reported signatures and more predictive of outcome than other clinical parameters used in the clinical practice [40].

In this work, we demonstrate for the first time an association between the expression of P-cadherin and HIF-1α in breast carcinomas, which was validated using *in vitro* breast cancer models. We showed that P-cadherin expression is modulated in a time-dependent way by hypoxia, since short-time incubations of BLBC cells in low oxygen

levels increased P-cadherin expression, whereas 24 hours of incubation of cells in these conditions, lead to a significant decrease of the expression levels of this protein. In our opinion, this transient increase of P-cadherin expression might be important to the initial induction of the aggressive response of breast cancer cells to hypoxia and be also partially responsible for the development of adaptation of breast cancer cells to the hypoxic environment. This opinion is also supported by the enrichment of *CDH3* transcripts in hypoxic conditions, found in the analysis of the online available GEP from Chen *et al.* study [315]. Moreover, it is know that in solid tumours, hypoxia is highly heterogeneous concerning its spatial distribution and intensity. Concerning kinetics, hypoxia is also highly diverse, since it is able to regulate several different cellular pathways with unique activation kinetics and sensitivity to oxygen concentration. However, most of the studies underlying the prognosis relevance of hypoxia signature in breast tumours do not take into account the time dependency of hypoxia-regulated gene expression. Interestingly, Seigneuric *et al.* studied the impact of the time-dependent response to hypoxia in the prognostic value of breast cancer patients [334]. By the analysis of gene signatures derived from either early or late hypoxic exposure of several primary cell lines, including a HMEC cell line, these authors observed that early hypoxia signatures show a significant prognostic power comparing to late hypoxia signatures, being the best prognostic factor in breast cancer after lymph node status, tumour size and Elston grade [334].

Thus, the recognition of P-cadherin as a poor prognosis marker in breast cancer, being significantly associated with short-term overall and disease free survival [228, 230, 277, 306], in addition to the data showing that early hypoxia-responses have increased prognosis value in breast cancer patients, are in accordance with our results showing that P-cadherin expression increases at early hypoxia exposure of breast cancer cells.

Besides the kinetics of hypoxia-induced signalling, it is also important to take into account that biological systems are extremely heterogeneous regarding oxygen concentrations: normoxia conditions of cell cultures *in vitro* is, by definition, the

oxygen tension of ambient air. However, oxygen concentrations between 2% and 9% have recently been reported by some authors to constitute physiologic normoxia [125]. Thus, it is important to set among experiments the most accurate oxygen pressure as possible, in order to more precisely reproduce the real tension experienced by a given cell *in vivo*. Although our *in vitro* results demonstrates that hypoxia modulates P-cadherin expression in a time dependent manner, implicating the kinetics of P-cadherin regulation by hypoxia, it would be also important to analyse P-cadherin modulation in different oxygen tensions that would reproduce hypoxia conditions observed in human breast carcinomas.

Further, and validating the association in breast carcinomas, as well as the enhanced P-cadherin expression in early hypoxia exposure times, we also find that HIF-1α stabilization promotes membrane P-cadherin expression in breast cancer cells upon $CoCl_2$ treatment. However, no alterations were detected in *CDH3* mRNA levels, which led us to hypothesize that increased P-cadherin membrane expression after HIF-1α accumulation occurs at a post-transcriptional level and not as a direct effect.

Thus, based in our findings and on previous data reported by others, the crosstalk between HIF-1α and P-cadherin might be explained by several hypotheses. One explanation can be related with the proteasome-dependent ER degradation induced by hypoxia through HIF-1α [49], observed in hypoxic/necrotic areas of breast carcinomas that show ER loss [49, 51]. Interestingly, ER negativity was already coupled to P-cadherin overexpression in breast carcinomas [249, 273]. In fact, in 2004, we have found that the antiestrogen ICI 182,780 (Faslodex) induces aberrant P-cadherin overexpression in breast cancer cells [241] by a mechanism involving chromatin remodelling of the gene promoter [246], identifying ER as a transcription *CDH3* repressor. Thus, hypoxia can be inducing HIF-1α, which promotes ER degradation and consequently P-cadherin increased expression. Other explanation can also be related to the existent link between hypoxia and BRCA1, which is also a *CDH3* transcriptional repressor [242]. It is known that hypoxia induces BRCA1 downregulation [335] and that *BRCA1*-mutated breast carcinomas, which are

enriched in HIF-1α expression [54], present aberrant expression of P-cadherin [247]. In addition, CAIX is also associated with somatic loss of BRCA1 protein and pathway activity in triple negative breast cancer [336]. Actually, Neumeister *et al.* demonstrated that CAIX expression was inversely related with nuclear BRCA1 expression in breast cancer patients and also that high CAIX protein expression occurs in patients who show the BRCA1 mutant signature and low levels of BRCA1 protein [336].

Finally, since it is also accepted that hypoxia, via HIF-1α, induces EMT with consequent E-cadherin loss of expression in different tumours models [337], including breast cancer [31], we can also hypothesize that P-cadherin increased expression, either in hypoxia and in $CoCl_2$ treated breast cancer cells, might be a consequence of hypoxia/ HIF-1α-induced E-cadherin downregulation. In fact, loss of E-cadherin has also been associated with increased aggressive behaviour in several human tumours, as well as with tissue dysfunction and cell death in early mouse embryos and in lactating mammary gland [338, 339]. Accordingly, our unpublished work shows that siRNA-mediated downregulation of E-cadherin leads to increased P-cadherin expression in breast cancer cell lines and it has also been shown that epidermal basal layer of skin respond to E-cadherin loss with an increase in P-cadherin membrane expression [340].

Interestingly, the HIF-1α-dependent gene product CAIX has also been associated with E-cadherin loss. In 2006, it was shown that VHL inactivation and HIF activation in precancerous lesions in kidneys from patients with VHL disease correlates with downregulation of E-cadherin [341], since portions of the distal tubules that expresses CAIX shows a substantial decrease in E-cadherin expression.

Concomitantly with P-cadherin increased levels, we also observed a decrease in the cellular height of breast cancer cells after $CoCl_2$ treatment, indicating a putative re-organization of the cytoskeleton, with the acquisition of a phenotype associated to breast cancer cell aggressiveness, such as increased ability of migration and motility [342]. This observation is consistent with our previous data, showing that P-cadherin expression is able to induce invasion, migration and motility of breast cancer cells

[295] and that its modulation also interferes with GTPase-mediated signal transduction and actin cytoskeleton organization [310].

After describing the significant associations in primary tumours, as well as the link between P-cadherin and HIF-1α transcription factor, either in breast carcinomas and *in vitro*, we found, using BLBC cell lines, that P-cadherin silencing is still able to induce the downregulation of *GLUT1* and *CAIX* mRNA, although *GLUT1* and *CAIX* knockdown showed little or no effect in *CDH3* mRNA expression. Because the expression variations were not so prominent as expected for a direct molecular target, we believe that P-cadherin is putatively involved in a signalling pathway that interferes with the metabolic reprograming of cancer cells. In accordance, we observed that the cell populations expressing more/less P-cadherin at the cell surface were the same presenting higher/lower levels of GLUT1 and CAIX and vice-versa. These results suggest that P-cadherin overexpressing breast cancer cells are most likely to exhibit increased glycolysis and to survive to metabolic-driven pH alterations, justifying the enhanced invasion and metastatic properties. Still, these concepts, as well as the above-discussed results, are reinforced by GEP analysis of breast cancer cells with *CDH3* deregulation, which revealed alterations in genes that regulate oxygen and nutrient levels. Accordingly, alterations in cyclic nucleotide metabolic processes, as well as in carboxylic acid transport processes, which are intimately associated with the adjustment of cells to nutrient and oxygen deprivation, were also found to be deregulated by P-cadherin expression in these GEP studies. Moreover, these results also strengthen the hypothesis that P-cadherin might have a role in the development of an advantageous phenotype of breast cancer cells, in order for them to survive and grow in adverse microenvironmental conditions.

P-CADHERIN, HIF-1α, GLUT1 AND CAIX AS CANCER CELL MARKERS IN BASAL-LIKE BREAST CANCER CELLS

Hypoxia and hypoxia inducible factors are implicated in tumourigenesis of several cancer models, including breast cancer. In 2006, Li *et al.* demonstrated that HIF-1α knockdown reduces tumourigenicity of breast cancer cells by reducing tumour growth in orthotopic and subcutaneous xenograft models and sensitizes cells to chemotherapy [343]. This effect is known to be attributed to the role of hypoxia in the expansion of a population of cancer cells with stem-like properties, the CSC [130]. Specifically in breast cancer, several reports have demonstrated that HIF-1α directly regulates breast CSC activity *in vitro* and *in vivo* [39, 46]. Using metastatic breast cancer cell lines, Louie *et al*. demonstrated that repetitive cycles of hypoxia and reoxygenation selects and expands a breast cancer cell population with high ability to form colonies *in vitro*, tumours in immune-deficient mice and with stem-like and EMT properties [39]. Recently, Schwab *et al.* also showed the pivot role of HIF-1α in the maintenance, expansion, as well as increased activity of breast CSCs, with implications in tumour growth and lung metastasis [46]. Moreover, it is accepted that the limited efficacy of anti-angiogenic therapies, which surprisingly demonstrated to increase invasive and metastatic properties of breast cancer cells, can be attributed to breast CSC expansion by tumour hypoxia. Using human breast cancer xenografts, Conley *et al.* demonstrated that anti-angiogenic factors, such as sunitinib and bevacizumab, are able to increase intratumoural hypoxia, which in turn leads to an increase of the breast CSC population, through the activation of Wnt pathway via Akt/β-ctn signalling [197]. Accordingly, Oliveira-Costa *et al.* also showed that HIF-1α was differently expressed in $CD44^{+}/CD24^{-/low}$ breast CSCs [194]. In addition to these reports, is has also been shown that CAIX also plays a role in the regulation of stemness and expansion of breast CSCs in hypoxic niches, since its expression and activity is required for the expansion of 4T1 $CD44^{+}/CD24^{-/low}$ cells in hypoxia [344]. Lock *et al.* showed that small-molecule mediated inhibition of CAIX expression or activity in breast cancer cell lines resulted in the inhibition of breast CSC expansion in hypoxia through the mTORC1 pathway [198]. *In vivo*, these authors also

demonstrated that the treatment of mice bearing orthotopic breast tumours with CAIX-specific small-molecule inhibitors results in a significant depletion of CSCs within these tumours. Accordingly, CAIX was shown to modulate the expression of EMT markers, as well as drivers of stemness such as Notch1 and Jagged1 in breast CSC [198]. Thus, CAIX is suggested to be a promising target for depleting CSCs from breast tumours, leading to improved chemotherapeutic response, since CAIX inhibitors, in combination with paclitaxel, decreased tumour growth and the formation of lung metastasis [198].

Recently, we have also demonstrated that P-cadherin expression is able to mediate stem cell properties in BLBC cells *in vitro*, which can be responsible for the aggressive behaviour of BLBC [306]. With P-cadherin gene expression manipulation, either by silencing with siRNA or by overexpression, as well as separation of P-cadherin^{high} and P-cadherin^{low} cell populations, we showed that this molecule induces stem cell activity and self-renewal of mammospheres, as well as clonogenic activity in 3D cultures of BLBC cells. We still demonstrated that P-cadherin-enriched cell populations have increased tumourigenic ability in athymic nude mice [306].

Taken together, all the described data indicate that all these molecules, which are representative of an adaptation to hypoxia with the acquisition of a glycolytic metabolism, are associated with stem-like properties in breast cancer cells. Accordingly, this work demonstrates that the inhibition of *CDH3, HIF-1α, GLUT1* and *CAIX* in BLBC cells significantly reduces their MFE, an important measure of breast CSC activity. We still show that the silencing of *CDH3* and *HIF-1α*, or even the simultaneous inhibition of *CDH3, HIF-1α, GLUT1* and *CAIX*, have a non-cumulative effect in the inhibition of MFE comparing to the single silencing of each one of these molecules. In a way, this result confirms our observation that the cell subpopulation expressing increased levels of P-cadherin at the cell surface was also the same presenting higher levels of GLUT1 and CAIX, demonstrating that we are targeting the same breast cancer cell population, which presents an increased stem cell activity.

Our results also demonstrated an association between glycolytic and acid-resistant markers with P-cadherin-related breast CSC markers, such as CD44 and CD49f. These molecules are putative mammary gland stem cell [152, 345, 346], as well as CSC markers of the basal phenotype [175, 178, 347], and are enriched in breast cancer cell lines with basal-like phenotype [179, 306, 348, 349], where P-cadherin is also known to be enriched. Besides the *in vitro* association found between P-cadherin expression and the breast CSC markers CD44 and CD49f, our group also reported the association of CD44 and CD49f with P-cadherin expression in human primary breast carcinomas [306]. Still, P-cad^{+}/CD44^{+} tumours present worse patient's survival, comparing with P-cad^{+}/CD44^{-} or with tumours with absence of P-cadherin expression [306].

Using immunohistochemistry in an independent series of primary invasive breast carcinomas, we were able to validate not only the above-described associations between P-cadherin with HIF-1α and GLUT1, as well as the association between P-cadherin, CD44 and CD49f expression, as previously reported [306]. Importantly, we found that GLUT1 expression was significantly associated with the expression of CD44 and CD49f, and CAIX expression was found to be significantly associated with CD44 expression. These results are in accordance with the concept that breast CSCs present an enhanced glycolytic phenotype [201]. Interestingly, CD44 was described as playing a role in the regulation of metabolism of glycolytic cancer cells [350]. Tamada *et al.* demonstrated that CD44 ablation reduces glucose uptake through the suppression of GLUT1 expression in p53 knockout cells *in vitro* [350].

Moreover, CAIX expression was already associated with the expression of CSC markers. Currie *et al.* studied the expression of CSC markers and its relationships with hypoxia and angiogenesis markers in breast carcinomas [329]. These authors found that CAIX expression was associated with the CSC marker CD133, but not with CD44^{+}/CD24$^{-/low}$ or with ALDH1 expression. Microvessel density, measured by CD31 immunoreactivity, was only associated with ALDH1 positive breast carcinomas[329].

HIF-1α is also a known regulator of CD44 expression in breast cancer cells. Krishnamachary *et al.* have demonstrated that hypoxia, via HIF-1α, induces the expression of the two CD44 variant isoforms, CD44v6 and CD44v8, in triple negative breast cancer cells *in vitro* [351]. They also showed an increased expression of CD44 in hypoxia regions of tumours *in vivo*, suggesting that increased expression of CD44 may be an additional mechanism by which hypoxia mediates a more aggressive phenotype and also reinforcing the presence of CSCs in hypoxic environments [351]. Moreover, other authors have also described a significant association between the expression of CD44 and HIF-1α, evaluated by immunohistochemistry in invasive breast tumours [352]. In contrast with these results, we were not able to find an association between HIF-1α and the P-cadherin associated CSC markers, CD44 and CD49f. Anyway, our previous results demonstrating that P-cadherin expression is able to modulate the expression of CD49f and CD44 in BLBC cells, as well as the above described studies, supporting the association between P-cadherin expression and the glycolytic and acid-resistance phenotype, corroborates the associations found between GLU1 and CAIX with breast CSC markers.

P-CADHERIN IS RESPONSIBLE FOR OXPHOS SUPRESSION IN BREAST CANCER CELLS

In this work we demonstrated, for the first time, that P-cadherin silencing was able to modulate cellular bioenergetics of SUM149 breast cancer cells, by allowing an increased OCR/ECAR rate, as well as cellular ATP content. These results suggest that P-cadherin is responsible for suppression of OXPHOS in these highly glycolytic IBC cells and that its expression influences the way by which cancer cells obtain their energy.

Several reports in the literature have been attempting to compare the metabolic profile of normal mammary tissue with breast tumours, implicating metabolic alterations in breast cancer progression [110-112, 353]. Most of these studies show an increased glycolytic activity and a decreased OXPHOS with cancer progression. Budczies *et al.* performed a metabolic map of breast cancer showing alterations in energy metabolism, catabolism of amino acids and nucleotide metabolism [113]. Furthermore, Richardson *et al.* also found key alterations in metabolic processes including PPP, TCA cycle, as well as synthesis of glutamate, glutathione and fatty acid upon breast cancer transformation, as well as alterations in *de novo* synthesis of glycine and proline in metastatic breast cancer cells [111]. Furthermore, a “two-step theory of breast cancer progression” was proposed by Lu *et al.,* also implicating metabolic deregulation in both breast tumourigenesis and progression [110]. Using a series of isogenic tumourigenic cell lines mimicking breast cancer progression, these authors demonstrated, by the quantitative analysis of metabolites involved in metabolic pathways, that fatty acid and nucleotide biosynthesis, glutathione antioxidant pathway, as well as glycolysis, TCA and PPP accompanies transformation and acquisition of metastatic potential of breast cancer cells [110]. Still, using the model of breast cancer progression of the immortalized mammary epithelial cell line MCF-10A, Shaw and co-workers demonstrated a decline of oxygen consumption rate in a series of cell lines mimicking breast cancer progression, thus proposing that it is accompanied by a decrease in cellular OXPHOS capacity [112]. Taken together, the above-presented studies implicate the deregulation of several metabolic processes in

breast cancer tumourigenesis and progression, which we also found to be altered in the analysis of GEP of breast cancer cells with modulation of P-cadherin expression. These results can be associated to the P-cadherin role in stemness maintenance, in the normal and cancer context. Kolle *et al.* have identified *CDH3* as one of the genes encoding a surface protein able to identify the pluripotent population of human ESC [255] and it expression was already reported in early progenitor cells from hair germs and small hair epidermal [257, 258], as well as in mammary stem cells [254]. In normal mammary gland, P-cadherin expression is confined to the myoepithelium, where adult stem cells are thought to exist. The same localization is also found in the development of the gland, where P-cadherin is observed in the precursor cell [230, 253-255]. Functionally, this adhesion molecule seems to be responsible for the maintenance of the undifferentiated state of the mammary gland, since the *CDH3-null* female mice exhibit precocious mammary gland differentiation in the virgin state, and breast hyperplasia and dysplasia with age [264]. Normal stem cells from several different tissues in the human body have distinct metabolic properties when compared to their more differentiated progeny [147, 354]. These undifferentiated cells use glycolysis as their main pathway to obtain ATP for their energetic demands, suggesting that increased glycolysis is a conserved stem cell property. It is believed that the hypoxic environment where stem cells reside, in stem cell niches, prevents differentiation being responsible for the maintenance of self-renewal and pluripotency of stem cells [2, 126]. ESC and iPSC exhibit similar bioenergetic metabolic profiles, redox status and mitochondrial function [355]. In fact, several reports have demonstrated that these cells exhibit enhanced glycolysis, as well as under-developed mitochondrial network and low mitochondrial activity compared with their more differentiated counterparts [147, 356-360]. Furthermore, Simsek *et al.* demonstrated that HSC present a unique metabolic profile, using glycolysis instead of oxidative phosphorylation, with low mitochondrial membrane potential and NADH levels, which provide these cells survival advantage during severe hypoxic or anoxic insults, which would eliminate more differentiated cells that rely on oxidative metabolism. Importantly, the transcriptional regulation of *HIF-1a* by Meis1 highlights the involvement of stem cell-associated transcription factors in the regulation of HSC

metabolism and indicates that the glycolytic phenotype of HSCs is not merely a product of their hypoxic environment [354]. In fact, hypoxic environments and mitochondrial inhibition have been associated with reduced stem cell differentiation and improved generation of iPSC [126, 128, 147]. Somatic cells undergo a metabolic shift in order to acquire ES cell-like features. Moreover, functionally active mitochondria are necessary for successful differentiation of ES cells, which requires an opposite switch in energy metabolism from anaerobic glycolysis to aerobic OXPHOS [133, 137]. Panapoulos *et al.* evaluated the bioenergetic alterations of somatic cell reprogramming, by the analysis of metabolomic and cellular bioenergetic of human iPSC relative to ESC and to their somatic cells of origin [118]. These authors found that somatic cells present a higher overall oxidative/glycolytic (by OCR/ECAR rates) compared to pluripotent stem cells, indicating that energy production of more differentiated cells is more dependent on oxidative phosphorylation than their pluripotent induced counterparts [118].

Due to the similarities of induced pluripotency and cancer, it is recognized that changes in cell metabolism that are important in the regulation of somatic cell reprogramming are also important in cancer [361]. Similar metabolism of normal stem cells is also observed in cancer cells with stem-like properties. The majority of the studies underlying the metabolic profile of CSCs are performed in gliomas [362-365]. GSC found in primary samples were found to have a pro-glycolytic phenotype [362-364]. In contrast, in other reports using glioma cell lines, GSCs and progenitor cells were found to be less glycolytic, consume less glucose, produce less lactate and present higher ATP levels than their differentiated progeny [365]. In this last study, GSCs and progenitor cells were found to use additional metabolic pathways, since inhibition of either glycolysis or oxidative phosphorylation had minimal effect on energy production [365].

Interestingly, breast CSCs are also described as presenting a glycolytic phenotype. Feng and co-workers demonstrated that CSC, from human and mice mammary tumours, use preferentially glycolysis instead of OXPHOS as their metabolic programme, when compared with non-breast CSC, where decreased expression and activity of mitochondrial PDH is being responsible for this metabolic behaviour [201].

These authors found that breast CSC present a higher ratio of lactate production to oxygen consumption, higher glucose consumption as well as fewer and less active mitochondria, than non-breast CSC [201]. The glycolytic dependence of breast CSC was further confirmed by the treatment of cells with metabolism-targeting drugs, where they concluded that oxidative phosphorylation promotion in breast CSC could represent a potential therapeutic strategy for targeting these aggressive breast cancer cells [201, 202]. The metabolic behaviour of breast CSC was recently confirmed by Ciavardelli *et al.*, using proteomic and targeted metabolomics analysis [202]. These authors demonstrated breast CSC shift from mitochondrial oxidative phosphorylation towards glycolytic metabolism and present increased expression of key enzymes of anaerobic metabolism, such as PKM2, LDH, and glucose-6- phosphate dehydrogenase, as well as increased antioxidant defence system [202]. Moreover, they observed that breast CSC treatment with 2-DG, an inhibitor of glycolysis, inhibits their proliferation when used alone and shows a synergic effect when used in combination with doxorubicin [202].

In conclusion, we suggest that inhibition of glycolysis may be a potentially effective strategy to target breast CSCs. Our results are concordant with the role of P-cadherin in the maintenance of stem-like properties in glycolytic breast CSC, as well as with the increased glycolytic and decreased oxidative phosphorylation associated with breast cancer progression. Since we demonstrate that P-cadherin inhibition in highly glycolytic breast cancer cells induces a metabolic shift towards oxidative metabolism, we hypothesize that this metabolic effect might be responsible for the decreased ability breast cancer cells to survive in anchorage independent condition upon *CDH3* silencing.

P-CADHERIN SUPRESSES OXIDATIVE STRESS IN BREAST CANCER CELLS

High rates of aerobic glycolysis are usually associated with increased proliferation and this phenotype is observed in rapidly proliferating cells, described as the "Warburg effect". However, stem and CSCs, which are relative quiescent, are described as having a glycolytic metabolic phenotype. For instance, HSC are more glycolytic, even though they are more quiescent than their differentiated counterparts [366]. Moreover, when comparing proliferating with quiescent fibroblasts, Lemons *et al.* observed that quiescent fibroblasts showed high glycolytic rates comparing to the proliferating ones [367]. Besides conferring fragility to Warburg hypothesis, these observations indicate that stem and CSCs undergo a metabolic glycolytic reprogramming independently of their cell cycle state. However, this phenotype is thought to be advantageous due to the diversion of metabolic intermediates into biosynthetic pathways, in order to meet the energetic demands for proliferation. Importantly, another advantage of this phenotype is the consequent ability to escape oxidative stress induced cell death. Oxidative stress occurs when production of reactive oxygen species (ROS) exceeds the capacity of the cellular defence systems, consisting of redox enzymes and other antioxidant molecules.

In this work, we observed a significant increase of ROS levels upon P-cadherin downregulation, as well as a decrease of ROS content upon P-cadherin overexpression in breast cancer cells.

At physiologic concentrations, ROS exerts essential signalling functions, being involved in the redox-dependent regulation of multiple signal transduction pathways, essential to biological processes, such cell adhesion, migration, proliferation, differentiation, and survival [204-207]. However, at high levels, ROS can induce damaging effects through oxidative stress. This is caused by an imbalance between the production of ROS and the ability of cellular antioxidant mechanisms to readily detoxify the reactive intermediates, making the maintenance of highly regulated mechanisms to control ROS levels and functional specificity, an essential process for

normal cellular homeostasis and cancer development**.** A glycolytic metabolism produces less ROS when compared with mitochondrial oxidative phosphorylation [11]. Thus, in accordance with their pro-glycolytic behaviour, several types of stem cells [211, 212] contain lower levels of ROS and manifest enhanced mechanisms for protection against ROS-mediated damage, comparing to their more mature progeny. Similarly, also cancer cells with stem-like properties have been described to present these same characteristics. In breast biology, normal mammary epithelial stem cells, as well as CSCs in human and murine breast tumours, contain lower ROS levels and increased scavenging systems [213, 217]. Particularly, Dihen *et al.* demonstrated that normal mammary stem cells, defined as a population enriched for mammary repopulating units, with the phenotype $CD44^{med}/CD49f^{high}Lin^{-}$, displayed low to intermediate levels of ROS, when compared to progenitor enriched populations [213]. Moreover, transplantation of these mammary stem cell populations according to their ROS levels, confirmed that mammary repopulation, a stem-like property, was associated with intracellular ROS, since these mammary stem cells sorted by their lower to intermediate ROS concentrations gave rise to epithelial outgrowth when transplanted in cleared mammary fat pad. Similarly, these authors also showed that, breast CSCs with $CD44^{+}/CD24^{-/low}/Lin^{-}$ phenotype presented higher levels of antioxidant defence associated genes, as well as lower levels of ROS [213].

Breast CSC are thought to persist in the tumour as a distinct population after therapy, causing relapses and metastasis, giving rise to new tumours. Along with other tumour microenvironmental factors, ROS are known to play a role in CSC formation, partially by the development of EMT. In fact, CSC exhibits increased expression of oxidative stress response genes, implicated in the survival of these cells to anticancer therapy [368, 369]. Emerging data have proven that high doses of ROS induce cell death in acute exposure, but the adaptation of cancer cells to this oxidative stress enables cells to survive and persist in the tumours. Interestingly, breast CSC are thought to be the most resistant pool of cells within the tumour bulk to therapeutical intervention, including radiation and oxidative stress inducing drugs [370]. ROS coming from mitochondria are responsible for anoikis-induced cell death in endothelial cells, as

well as in CSC, and the anoikis-resistance observed in CSC are thought to be due to lower ROS levels and enhanced scavenging systems observed in these cells [371]. Thus, the glycolytic behaviour of CSC as well, as their reduced OXPHOS, enabling low production of ROS, constitutes a survival advantage to breast CSC in their hypoxic niches, as well as in the exposure to hypoxic/anoxic episodes in the tumour microenvironment. Moreover, cell death induced by radiotherapy and chemotherapeutic drugs used in breast cancer, such as cisplatin, Adriamycin and 5-Fluorouracil, are thought to be mediated by the lethal effect of ROS [216, 372-376]. Thus, chemo and radioresistance exhibited by CSCs (Phillips de mama, Dallas chemoresistance colorectal cancer research 2009) have been attributed to their low levels of ROS and enhanced ROS defences. Survival of CSCs after ionizing radiation has been largely documented in brain tumours [377], as well as in breast CSC [213, 217]. Mice bearing MMTV-*Wnt*-1 tumours treated with short fractioned ionizing radiation showed a two-fold increase in the percentage of breast CSC comparing with non-breast CSC after irradiation, confirming the radioresistance of CSC in breast tumours. Accordingly, using breast cancer cell lines, Phillips and colleagues also reported that breast CSC represent a radioresistant subpopulation of breast cancer cells that are expanded after short courses of fractionated irradiation [217]. In this study, the authors observed a higher survival and lower ROS content in breast cancer cells growing in non-adherent conditions (representative of breast CSC), compared to cells growing in monolayer, after short courses of fractionated irradiation [217].

Recently, an interesting major role has been attributed to ROS in both outside-in and inside-out signalling of integrins, cadherins and small GTPases, raising the possibility that ROS constitute master regulators of the crosstalk between fundamental cell adhesion receptors [378]. Interestingly, our previous and ongoing work has been disclosing important knowledge about P-cadherin, a poor prognosis factor in breast cancer, as a pro-invasion molecule when co-expressed with E-cadherin, promoting cell-ECM attachment and alterations in the actin cytoskeleton, by increasing the expression of the laminin receptor α4β6 integrin, inducing p120-ctn cytoplasmic delocalization, and the activation of Src, FAK, AKT, Rac1 and MMP1/2[295, 296,

308]. Taken together, these findings reinforce the observed role of P-cadherin expression in ROS signalling.

Many studies have been trying to understand the mechanism for adaptation to ROS-induced toxicity leading to acquired chemotherapeutic resistance in breast cancer cells. Interestingly, Mahalingaiah *et al.* demonstrated that persistent exposure to oxidative stress increases tumourigenic and metastatic potential of breast cancer cells. These authors observed that, while acute exposure to H_2O_2-induced ROS inhibits the growth of MCF-7 breast cancer cells, chronic exposure leads to increased cell growth and survival, with increased number of soft agar colonies, upregulation of pro-metastatic genes *VEGF*, *WNT1* and *CD44*, as well as to downregulation of E-cadherin [379]. Interestingly, E-cadherin loss and EMT have been widely associated with enhanced radioresistance in breast cancer cells. Using clonogenic survival experiments in breast cancer cell lines, Theys *et al.* have demonstrated that low expression of E-cadherin confers resistance to radiation in breast cancer cells and reintroduction of E-cadherin sensitize cells after irradiation [380]. Moreover, Mori *et al*. also showed a decreased E-cadherin expression and enhanced expression of MMPs in mouse mammary gland epithelial cells, when exposed to oxidative stress for two to four days, with consequent enhanced invasive potential [381]. Interestingly, it is also reported that oestrogen-induced ROS is associated with E-cadherin downregulation [382]. However, besides its tumour/invasion suppressor function, the “dark side” of E-cadherin has already been described [383], and the regulation of energy metabolism is an important part of this aggressive behaviour [384]. Loss of E-cadherin and overexpression of E-cadherin regulators, such as ZEB1, in highly aggressive breast cancer cells, was shown to reduce tumour growth and metastasis formation, and gene profiling analysis demonstrated that loss of hypoxia response of IBC cells was the responsible mechanism for these observations [384]. In this study, Chu *et al.* demonstrated that E-cadherin silencing reduced tumour growth and this effect was rescued by HIF-1α expression; in addition, they demonstrated that E-cadherin expression downregulates the expression of HIF-1α, as well as some of its targets genes, such as CAIX. Accordingly, they also observed a reduction of lactate production and ECAR of IBC cells when E-cadherin expression was silenced,

showing that E-cadherin promotes glycolytic behaviour of IBC cells [384].
Tamada *et al.* have demonstrated that CD44 modulates metabolism of cancer cells, promoting glycolysis by the interaction with PKM2 in cancer cells that are either deficient in p53 or exposed to hypoxia, suggesting that CD44 is a potential therapeutic target for glycolytic cancer cells that manifest drug resistance [350]. Using siRNA-mediated knockdown, these authors showed that CD44 downregulation induces a metabolic shift towards mitochondrial respiration, with increased metabolic flux to TCA and concomitantly decreased entry into glycolysis and the PPP. These effects were also accompanied by a decrease in ATP cellular content as well as an increased the intracellular ROS levels. Furthermore, they also showed that treatment of cancer cells with mitochondrial respiration enhancer, DCA, have the same effect as CD44 decreased expression in the metabolic shift observed in glycolytic cancer cells. Interestingly, they also found that CD44 ablation enhanced the chemotherapy-induced cell death, probably by the increase in ROS levels [350].

The unique metabolic programme of aggressive breast CSC, like their glycolytic behaviour, reduced ROS levels and increased antioxidant defences, which enables these cells to survive to radiation and chemotherapy-induced cell death, is an extremely attractive therapeutical strategy for the development of breast CSC target drugs. Thus, oxidative stress modulation has been suggested as an effective strategy to target CSC and to increase radio and chemotherapy efficiency. For example, 2-DG, a glucose analogue, known to decrease glycolytic metabolism, has been used in cancer diagnosis and treatment. *In vitro* studies have shown that this compound inhibits cancer cell growth in several cancer models [385, 386]. Interestingly, breast CSC were found to be more sensitive to 2-DG treatment than their non-CSC counterparts [201], alone or in combination with chemotherapic drugs such as doxorubicin [202]. However, the clinical success of this compound was compromised due to its increased autophagy and toxicity to the brain and heart. Another example is 3-BromoPyruvate (3-BrP), which although is not in any clinical trial, it has been considered as a potential cancer therapeutic agent due to its effects on different cancer types, including breast cancer [387-390]. Finally, DCA, a pyruvate analogue

that inhibits pyruvate dehydrogenase kinase (PDK), diverting pyruvate to mitochondrial Krebs cycle [162, 391, 392], has been also target of several studies that stressed out the possible role of DCA as a potential anti-cancer drug for several types of cancer, including breast cancer [203, 393, 394], due to its anti-proliferative and pro-apoptotic potential. Interestingly, DCA seems to play an important role in cell death induction, specifically in CSC. *In vitro* and *in vivo* studies have been shown that DCA treatment inhibits proliferation of glioblastoma stem-like cells [395]. In breast cancer, DCA was also found to decrease breast CSC clonogenicity in 3D spheroid culture systems in a dose dependent manner [201]. This effect seems to be related with the decreased expression and activity of PDH found in breast CSC when compared with non-breast CSC [201]. DCA was already on the base of one phase II clinical trial [395] and another trial with DCA in currently on going ('ClinicalTrials.gov').

P-CADHERIN MODULATES ANTI-OXIDANT PROPERTIES IN BREAST CANCER CELL

ROS are mainly produced in the mitochondria and the accumulation of these molecules is controlled by the potent anti-oxidant machinery of the cell. Antioxidants seem to have dichotomous activities in tumourigenesis. By one side, they suppress tumourigenesis by preventing oxidative damage to DNA [396]. By the other side, they promote tumourigenesis by allowing survival of cancer cells. In fact, increased antioxidant activity has been reported as playing an important role in breast cancer progression. Lu *et al.* reported a reduced anti-oxidant activity in tumourigenic and metastatic cells lines, suggesting that aggressive behaviour of breast cancer cells is associated with decreased ROS levels and increased anti-oxidant defence systems [110]. Furthermore, a recent study has revealed that enhanced PPP flux and increased antioxidant capacity correlates with metastasis of breast cancer cells to the brain [397].

The first line of defence of ROS-induce DNA-damage is the activity of mitochondrial isoenzymes SODs, which main function is to dismutase superoxide anion into H_2O_2 and O_2. MnSOD (SOD2) reduces oxidative stress caused by respiratory chain leak and is responsible for the detoxification of ROS in the mitochondrial matrix [398]. Either decreased or increased expression of SOD2 has been reported, comparing to their normal counterparts, depending on the type and grade of tumour [399-403]. In breast cancer, SOD2 expression is dependent of ER status and associated with invasive and metastatic properties of breast cancer cells. ER positive cell lines exhibit low SOD2 expression, in contrast with MCF-10A and HMEC, while high levels of expression are present in oestrogen independent and metastatic breast cancer cells [404, 405]. In human breast tumours, SOD2 is decreased in *in situ* breast carcinomas and in benign hyperplasias [406], while in invasive tumours, its expression is found to be increased, specially in advanced, high grade and poorly differentiated tumours [406-408]. Moreover, it was also reported that micro and macro-metastasis presented higher levels of SOD2 when compared to their matched primary breast tumour [409]. Still, high SOD2 is associated with hormone receptor negativity, p53 mutations,

decreased disease free survival, as well as with high histological grade in breast cancer samples [409]. However, other reports have been describing the decreased SOD2 expression in invasive breast carcinomas. The expression and activity of this antioxidant protein is regulated by deacetylase sirtuin-3 (SIRT3) [410], which is found to be lost or decreased in 87% of breast cancers [411]. Since SIRT3 is able to modulate the activity of SOD2 [410], being responsible for the increase of ROS levels in cancer cells [412], SIRT3 downregulation is essential for the metabolic reprograming toward glycolysis, through the stabilization of HIF-1α [410]. Based on this increased ROS levels due to SOD2 downregulation, cancer cells were found to upregulate SOD1 in order to retain low levels of superoxide and avoid irreversible damage and allow cell survival, known as the "SOD2 to SOD1 switch". Overexpression of SOD1 is able to promote growth and survival of different types of cancer, including breast cancer. In fact, increased SOD1 expression is a frequent event in breast cancer. Papa *et al.* demonstrated that loss of SOD2 correlates with the overexpression of SOD1, either in breast cancer cells lines as well as in human breast carcinomas, suggesting that SOD1 increase in breast cancer cells with SOD2 downregulation is a mechanism for breast cancer cell survival [413]. Moreover, mammary tumour models present high levels of SOD1 protein, while normal mammary gland does not exhibit the expression of this protein [413]. Accordingly, it is reported that LCS-1, a molecule that target SOD1 activity, leads to mitochondrial damage and inhibits breast cancer cell growth *in vitro,*supporting the hypothesis that SOD1 is essential for adaptation of breast cancer cells to oxidative stress [413].

In this work, we demonstrated that P-cadherin expression is associated with low ROS content, probably by the induction of ROS scavenging systems, such as SOD1 expression and SOD2 activity (superoxide dismutase 1 and 2). Conversely, we also observed that inhibition of P-cadherin expression increases ROS levels with concomitant decreased SOD antioxidant systems. These results are in agreement with our GEP analysis of breast cancer cells with P-cadherin deregulation, which revealed alterations in gene expression associated with antioxidant mechanisms, such as glutathione metabolism. Moreover, these results are also in agreement with

our previously reported findings, also observed in this work, showing the increased caspase-dependent cell death in P-cadherin^{low} breast cancer cells populations, as well as with the increased anoikis observed in P-cadherin^{low} versus P-cadherin^{high} cells. Accordingly, our GEP analysis also suggests that P-cadherin expression is involved in mitochondrial apoptotic defects, which are crucial for oncogenesis and resistance to therapy and can be associated with metabolic reprogramming of breast cancer cells.

Interestingly, cellular adaptation to oxidative stress is recognized to be a mechanism of intrinsic and acquired resistance to several anticancer drugs, since antioxidant capacity is one of the classical stress response mechanisms that modulate apoptosis. For instance, lapatinib, a drug targeting epidermal growth factor receptors 1 and/or 2 (EGFR/HER2), has been used in treatment of IBC, triple negative breast carcinomas which typically present overexpression or activation EGFR/HER2. However, a significant number of patients have demonstrated acquired resistance to lapatinib, despite the decrease in EGFR/HER2 phosphorylation and downstream signalling activation [414-416]. Aird *et al.* analysed the role of oxidative stress adaptation in lapatinib-analogue (GW583340) sensitive and resistant IBC cell lines and demonstrated that, cells with acquired resistance to this compound, presented high levels of SOD1 and SOD2, as well as glutathione content, with no alterations in ROS levels, when comparing to sensitive cells [417]. They also observed that modulation of SOD activity in GW583340-resistant cells was able to induce apoptosis in the presence of ROS generators, implicating SOD and antioxidant mechanism deregulation in cell survival, responsible for resistance to lapatinib in the aggressive form locally advance breast cancer [417]. Accordingly, Williams *et al.* also demonstrated an increased expression of SOD2, as well as an increase of pro-survival factors such as XIAP and Bcl-2, in SUM149 cells with resistance to lapatinib [418]. These authors observed that reverted resistance to lapatinib in IBC cells, by drug removal, resulting in a re-sensitization to multiple drugs, was accompanied by a decrease of SOD2 expression, increased ROS levels, increase p-AMP, indicators of an enhanced oxidative stress, as well a decreased in the pro-survival machinery

[418].

Moreover, the vast majority of studies focuses on bulk population of cells and do not take into account the heterogeneity of the tumours. Given that this heterogeneity is also reflected in a distinct metabolic behaviour of cancer cells, it is important to combine target therapies targeting different aspects of metabolism in order to obtain more dramatic effects in the clinical outcome. The failure of metabolic target drugs can be explained by the action of these compounds in CSC, which comprises only a small population of cells within the tumours. For this reason, it is extremely important to focus the effect of the drugs specifically in breast CSC.

Cancer cells develop resistance to anoikis, defined as the induction of apoptosis due to the detachment from the ECM, allowing tumour progression and metastasis [419, 420]. Matrix detachment of normal mammary epithelial cells markedly stimulates the generation of ROS, leading to induction of anoikis [421, 422]. However, in order to counteract the deleterious effect of ROS, these cells develop mechanism to prevent accumulation of ROS. Oxidative metabolism and stress response are know to play a major role in the survival of normal mammary epithelial cells as well as breast CSCs. It is described that normal mammary epithelial cells attenuates glucose oxidative metabolism [215] and also enhances antioxidant ability to detoxify ROS, lowering oxidative stress and prolonging survival in suspension [409]. Schafer *et al.* demonstrated that detachment of mammary epithelial cells from ECM causes an ATP deficiency due to loss of glucose transport, which could be rescued by overexpression of HER2 by restoring glucose uptake through stabilization of EGFR and PI3K activation [422]. Moreover, Kamarajugadda *et al.* demonstrated that, in response to matrix detachment, mammary epithelial cells inhibit oxidative metabolism of glucose and increase SOD2 expression, conferring them anoikis-resistance, and also that depletion of SOD2 expression sensitizes these cells to anoikis by the increased ROS content [409]. Cancer cells already exhibit a metabolic program with reduced glucose oxidation, which allows the maintenance of low ROS levels and escape to anoikis. ECM-detached breast CSC exhibit a specific pro-glycolytic

behaviour and an enhanced ROS scavenging systems [110, 201], which allows them to decrease oxidative stress, being able to escape anoikis, survive in circulation and enhance metastasis formation. Thus, inhibition of ROS detoxification coupled with stimulation of glucose oxidative metabolism may be an efficient strategy to enhance anoikis, and since this type of cell death is a barrier to breast cancer metastasis, it is probably a good therapeutical approach to impair breast cancer metastasis. Importantly, P-cadherin expression promotes stem-like properties to BCC, such as tumourigenic capacity and anoikis-resistance, allowing cells to survive in anchorage-independent conditions [306]. In addition, P-cadherin also conferred radiation-resistance to breast cancer cells and, upon apoptotic stimuli, decreased P-cadherin expression increases breast cancer cell death in a caspase-dependent mechanism [296, 306].

Taken together, we hypothesize that the role of P-cadherin in oxidative stress can be responsible for anoikis-resistance observed in breast CSC. Still, the obtained data suggests that P-cadherin might be a player in the radiotherapy resistance through the metabolic regulation of breast CSCs, due to its role in the adaptation to oxidative stress.

CHAPTER VI

Conclusions and Future Perspectives

Conclusions

The data presented and discussed in this thesis allowed us to conclude that:

1. Aberrant P-cadherin expression is associated with the hypoxic/glycolytic and acid-resistant phenotype in invasive breast carcinomas. Using immunohistochemistry, we were able to prove that breast carcinomas with HIF-1α, GLUT1, CAIX, MCT1 and CD147 expression show a high percentage of P-cadherin positivity.

2. The expression of P-cadherin is associated with HIF-1α in breast cancer. Primary invasive breast tumours with P-cadherin aberrant expression also exhibit an enrichment of HIF-1α expression. Moreover, hypoxia/HIF-1α accumulation in breast cancer cells *in vitro* is able to induce an increased membrane P-cadherin expression in basal-like breast cancer cells.

3. P-cadherin regulates and is co-expressed with effectors of the metabolic reprograming, such as GLUT1 and CAIX, in basal-like breast cancer cell populations with increased ability to survive in anchorage-independent conditions.

4. Glycolytic and acid resistance phenotypes are associated with the expression of P-cadherin-related breast CSC markers in human breast carcinomas. This suggests that the stem-like properties of P-cadherin-enriched breast cancer cells can be associated with the P-cadherin role in breast cancer cell metabolism.

5. P-cadherin expression is responsible for the maintenance of the glycolytic behaviour of aggressive IBC. Decreased expression of P-cadherin induces a metabolic shift towards oxidative phosphorylation, with an increase in energy production in inflammatory/basal-like breast cancer cells.

6. Oxidative stress is modulated by P-cadherin expression in breast cancer cells**.** ROS content as well as antioxidant systems, SOD1 and SOD2, are modulated by P-

cadherin expression in breast cancer cells, which might be responsible for cell survival of P-cadherin-enriched breast CSC populations.

Considering the initial aims of this PhD project, we believe that we have successfully addressed most of the questions we have proposed to answer. In conclusion, the results presented in this study highlighted P-cadherin as a relevant molecule for breast cancer metabolism.

Future perspectives

It is now clear that aggressive behaviour induced by P-cadherin in breast cancer cells might be mediated by its implications in cellular metabolism. However, the data resulting from this work raises many interesting questions and challenges that remain to be elucidated. In order to better understand the mechanism and the resulting implications of P-cadherin's regulation of metabolism, we aim to develop the knowledge on metabolomics and mitochondrial functionality in breast cancer cells. Moreover, the results from this work also led us to believe that P-cadherin expression might be mediating anoikis-resistance by the modulation of cellular metabolism and oxidative stress, being responsible for the survival of breast CSC and consequently for the development of breast cancer metastasis. Thus, additional questions should be addressed in future studies:

1. To understand how P-cadherin expression specifically influences the metabolic flux of basal-like BC cells.

2. To better comprehend the expression and activity of the glycolytic and OXPHOS machinery specifically involved in P-cadherin signalling.

3. To evaluate the effect of P-cadherin expression in the metabolic profile of breast CSC, in anchorage-independent conditions.

4. To evaluate the mitochondrial biogenesis, complexity and function mediated by P-cadherin expression in breast CSC.

5. To understand the oxidative stress suppressor role of P-cadherin in anoikis-resistance of breast CSC with enriched P-cadherin expression.

6. To evaluate the effect of targeting anoikis-resistant P-cadherin-enriched breast CSCs by *in vitro* metabolic reprograming, using metabolic targeting drugs, such as DCA.

7. To examine the putative synergistic effect of P-cadherin silencing and metabolic targeting drugs, such as DCA, in the effective elimination of P-cadherin-enriched breast CSC *in vitro*.

8. To evaluate the potential synergistic effect metabolic reprograming by targeting glycolytic breast CSCs with DCA, as well as with P-cadherin silencing, in breast cancer metastasis, using *in vivo* models.

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APPENDIX

PUBLICATIONS

Paper 1

BMC Cancer

RESEARCH ARTICLE Open Access

The basal epithelial marker P-cadherin associates with breast cancer cell populations harboring a glycolytic and acid-resistant phenotype

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Abstract

Background: Cancer stem cells are hypoxia-resistant and present a preponderant glycolytic metabolism. These characteristics are also found in basal-like breast carcinomas (BLBC), which show increased expression of cancer stem cell markers.
Recently, we demonstrated that P-cadherin, a biomarker of BLBC and a poor prognostic factor in this disease, mediates stem-like properties and resistance to radiation therapy. Thus, the aim of the present study was to evaluate if P-cadherin expression was associated to breast cancer cell populations with an adapted phenotype to hypoxia.

Methods: Immunohistochemistry was performed to address the expression of P-cadherin, hypoxic, glycolytic and acid-resistance biomarkers in primary human breast carcinomas. *In vitro* studies were performed using basal-like breast cancer cell lines. qRT-PCR, FACS analysis, western blotting and confocal microscopy were used to assess the expression of P-cadherin after HIF-1α stabilization, achieved by $CoCl_2$ treatment. siRNA-mediated knockdown was used to silence the expression of several targets and qRT-PCR was employed to evaluate the effects of P-cadherin on HIF-1α signaling. P-cadherin high and low breast cancer cell populations were sorted by FACS and levels of GLUT1 and CAIX were assessed by FACS and western blotting. Mammosphere forming efficiency was used to determine the stem cell activity after specific siRNA-mediated knockdown, further confirmed by western blotting.

Results: We demonstrated that P-cadherin overexpression was significantly associated with the expression of HIF-1α, GLUT1, CAIX, MCT1 and CD147 in human breast carcinomas. *In vitro*, we showed that HIF-1α stabilization was accompanied by increased membrane expression of P-cadherin and that P-cadherin silencing led to a decrease of the mRNA levels of *GLUT1* and *CAIX*. We also found that the cell fractions harboring high levels of P-cadherin were the same exhibiting more GLUT1 and CAIX expression. Finally, we showed that P-cadherin silencing significantly decreases the mammosphere forming efficiency in the same range as the silencing of HIF-1α, CAIX or GLUT1, validating that all these markers are being expressed by the same breast cancer stem cell population.

Conclusions: Our results establish a link between aberrant P-cadherin expression and hypoxic, glycolytic and acid-resistant breast cancer cells, suggesting a possible role for this marker in cancer cell metabolism.

Keywords: P-cadherin, Breast cancer, Hypoxia, Cancer stem cells

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Background

The tumor microenvironment is markedly defective on oxygen and nutrients, which seems to have a huge influence on the selection and survival of cancer stem cell populations. In fact, it is widely accepted that developing embryos, as well as regions of tissues with cells harboring stem cell properties (stem cell niches), usually present low oxygen tensions, suggesting hypoxia as a crucial event to maintain the undifferentiated state of stem/progenitor cells [1,2]. Additionally, it is already widely accepted that undifferentiated cells, such as human embryonic stem cells (hESC) and induced-pluripotent stem cells (iPSC), present a glycolytic phenotype, decreased oxidative phosphorylation and ROS (reactive oxygen species) production, as well as altered lipid metabolism, when compared to their normal differentiated counterparts [3].

Cancer cells with stem-like properties, also known as cancer stem cells (CSC) or tumor-initiating cells (TICs), are also thought to reside in hypoxic niches within the tumor [2], exhibiting a metabolic program that allows their survival in this aggressive microenvironment [3]. This metabolic reprogramming is now recognized as a hallmark of cancer [4] and several players involved in cell metabolism are currently being considered as targets for cancer therapy [5].

HIF-1α, either induced by hypoxia or by alterations in oncogenes and/or tumor suppressor genes, induces the expression of gene products responsible for mediating changes in energy metabolism, pH regulation, angiogenesis, survival, invasion and motility [6]. In breast cancer, HIF-1α expression is associated with high-grade tumors, loss of estrogen receptor (ER), increased proliferation levels, decreased disease-free and overall patient survival and also with chemo- and radiotherapy resistance [7]. Moreover, cancer cells usually require increased glucose consumption, achieved by enhanced expression of glucose transporters (such as GLUT1). The increased glycolysis leads to intracellular acidosis that is controlled by upregulation of other membrane transporters, such as carbonic anhydrase IX (CAIX), monocarboxylate transporters (MCT1 and MCT4) and CD147/EMMPRIN (an extracellular matrix metalloproteinase inducer, required for proper location and function of MCT1 and MCT4).

Interestingly, the adaptation of cancer cells to limited oxygen availability, altered glucose metabolism and extracellular acidosis, are linked to a poor patient prognosis in breast cancer [8-10]. Chen *et al.* demonstrated that hypoxia, glycolytic and acid-resistant phenotypes are a powerful tumor cellular advantage and are associated to an aggressive behavior of breast carcinomas [11]. Moreover, several studies have been reporting that basal-like breast carcinomas (BLBC) show a stronger response to hypoxia [8,10], as well as a higher glycolytic metabolism, than tumors with luminal characteristics [12-16]. In fact, triple-negative and HER2-overexpressing breast carcinomas present the higher tissue glucose metabolism, measured by 18 F-FDG PET scan, in comparison with the other breast cancer molecular subtypes [17], reinforcing the association between glycolytic metabolism and breast cancer poor prognosis.

P-cadherin, a calcium dependent cell-cell adhesion molecule encoded by the *CDH3* gene, is a protein whose expression is highly associated with undifferentiated cells in normal adult epithelial tissues, as well as with poorly differentiated carcinomas [18]. Its expression has been already reported in hESCs and is presumed to be a marker of stem or progenitor cells in epithelial tissues [19,20]. During normal breast development, P-cadherin has a crucial role in the ductal mammary branching, being expressed by the monolayer of epithelial cap cells at the terminal end buds (TEBs) [21]. Moreover, this molecule is important for the undifferentiated state of the normal mammary gland [19], with its expression being restricted to the myoepithelium, although it has been postulated that it may be also present in early luminal progenitor cells [18,22,23]. In breast cancer, P-cadherin is aberrantly expressed in high-grade *in situ* [24] and invasive tumors [25], being a well-established indicator of poor patient prognosis [18,22,26]. The expression of P-cadherin is significantly associated with basal-like molecular subtype [25], which is mainly characterized by a triple-negative phenotype [ER, progesterone receptor (PgR) and HER2 negativity] and by the expression of basal/myoepithelial markers [27].

Moreover, P-cadherin expression promotes oncogenic-associated effects in breast cancer [18,22]. Using *in vitro* and *in vivo* models, we demonstrated that it induces tumorigenesis, as well as cancer cell invasion partially through the secretion of matrix metalloproteinases (MMPs), such as MMP1 and MMP2 [28]. We have also disclosed that P-cadherin functional role is dependent on E-cadherin cellular context, since it interferes with the endogenous cadherin/catenin complex, inducing p120-catenin cytoplasmic delocalization and the consequent associated alterations in the actin cytoskeleton [29].

Recently, our group demonstrated that P-cadherin expression is associated with breast cancer stem cell markers, namely CD44, CD49f and ALDH1 [30]. We revealed that highly tumorigenic P-cadherin-enriched breast cancer cell populations harbor increased survival, resistance to radiation and stem cell properties [30]. Additionally, since it is accepted that breast cancer stem cells are pro-glycolytic [31] and more resistant to radiotherapy regimens [32], we hypothesized that the expression of P-cadherin could be associated to cell populations with an adapted phenotype to hypoxia and altered metabolism. Interestingly, by the analysis of an online available gene expression profile (E-GEOD-9649) [9], we could observe

that *CDH3* gene is indeed upregulated in hypoxia compared to normoxic conditions, as well as in response to lactic acidosis.

In this work, we demonstrate that there is a significant association between aberrant P-cadherin expression and hypoxic, glycolytic and acid-resistant breast cancer cells, suggesting a role for this epithelial basal marker in cancer cell metabolism.

Methods

Breast tumor samples

Formalin-fixed, paraffin-embedded tissues of 473 invasive breast carcinomas in Tissue Microarrays (TMAs) were retrieved from the histopathology files of three Departments of Pathology: University Hospital of the Federal University of Santa Catarina (UFSC, Florianópolis, Brazil), Hospital Divino Espírito Santo (Ponta Delgada, Portugal) and from a private Laboratory of Pathology (Veronese Patologia e Citologia Araçatuba, Brazil). The tumors have been characterized for clinical and pathological features (data are summarized in Table S1 in Additional file 1). Molecular characterization of this series was previously studied and described [27]. This study was approved by the UFSC Ethics Committee for Human Research (CEPSH), by the Ethics Committee for Health from the Hospital do Divino Espírito Santo de Ponta Delgada E.P.E., as well as by the research review boards from the Veronese Patologia e Citologia Araçatuba Pathology Laboratory. Patients have signed a written informed consent, which implies that the spare biological material, which has not been used for diagnosis, can be used for research. This is in accordance with the national regulative law for the handling of biological specimens from tumor banks, being the samples exclusively available for research purposes in retrospective studies, as well as under the international Helsinki declaration.

Cell culture

Human breast cancer cell lines were obtained as follows: BT20 was acquired from American Type Culture Collection (Manassas, VA, USA) and SUM149 was kindly provided by Dr. Stephen Ethier (University of Michigan, USA). Cells were routinely maintained at 37°C, 5% CO_2, in the following media (Invitrogen Ltd, UK): DMEM for BT20 and 50% DMEM/50% Ham-F12 for SUM149. In BT20, the media contained 10% heat-inactivated fetal bovine serum, FBS, (Greiner bio-one, Belgium) and in SUM149 cell line, media was supplemented with 5% FBS, 5 μg/ml of insulin and 1 μg/ml of hydrocortisone (Sigma-Aldrich, USA). All media were supplemented with 100 IU/ml penicillin and 100 mg/ml streptomycin (Invitrogen Ltd, UK).

Primary antibodies

The following primary anti-human antibodies were used for western blot (WB), immunohistochemistry (IHC) and flow cytometry (FC) against: P-cadherin [clone 56, BD Transduction Biosciences, USA; diluted 1:500 (WB) and 1:50 (IHC)], and APC-conjugated P-cadherin, R&D, USA; diluted 1:10 (FC)], HIF-1α [clone 54, BD Transduction Biosciences, USA; diluted 1:500 (WB) and 1:50 (IHC)], CAIX [ab15086, AbCam, Cambridge, UK; diluted 1:1000 (WB), 1:2000 (IHC) and 1:10 (FC)], Glut1 [ab15309, AbCam, Cambridge, UK; diluted 1:400 (WB), 1:500 (IHC) and 1:10 (FC)], MCT1 [AB3538P, Chemicon International, USA; diluted 1:200 (IHC)], MCT4 [AB3316P, Chemicon International, USA; diluted 1:100 (IHC)], CD147 [18-7344, Zymed Laboratories Inc., USA; diluted 1:750 (IHC)], GAPDH [0411, Santa Cruz Biotechnologies, USA; diluted 1:10000 (WB)] and β-actin [clone I-19, Santa Cruz Biotechnologies, USA; diluted 1:1000 (WB)].

Immunohistochemistry

The immunohistochemical assays were performed with specific antibodies for P-cadherin, HIF-1α, GLUT1, CAIX, MCT1, MCT4 and CD147. Details about experimental procedures, primary antibodies, antigen retrieval detection systems and scoring are described elsewhere [12,13,27]. Specifically, HIF-1α immunohistochemistry was performed using CSA, Catalyzed Signal Amplification System (DAKOCytomation, USA), according to manufacturer's instructions. Reactions were independently evaluated by two pathologists (FS and RG). All the proteins showed membrane staining, consistent with their cellular function, except for HIF-1α, which presented a nuclear pattern of expression and was considered positive whenever any strong and dark nuclear staining was observed. For the different antibodies studied, some samples could not be evaluated for the 473 cases of the series due to TMA's cores missing or to insufficient representation of the tumor in the TMA core.

siRNA transfection

Gene silencing was performed with validated siRNA, specific for *CDH3* (50nM, Hs_*CDH3*_6), HIF-1α (50nM, Hs_HIF1A_5), *GLUT1* (100nM Hs_*SLC2A1*_2) and *CAIX* (50nM, Hs_*CA9*_2). All siRNAs were from Qiagen (USA). Transfections were carried out using Lipofectamine 2000 (Invitrogen, UK), according to manufacturer's recommended procedures. After incubation for 5 minutes, the siRNA and Lipofectamine 2000 solutions were mixed, incubated for additional 20 minutes and added to cell culture medium. A scrambled siRNA sequence, with no homology to any gene, was used as a negative control (Qiagen, USA). Gene inhibition was evaluated after 48 hours of cell transfection for *CDH3,* HIF-1α and *GLUT1* and after 72 hours for *CAIX*.

$CoCl_2$ treatment

Breast cancer cells were plated in T25 flasks for qRT-PCR and FACS, or in coverslips for immunofluorescence. After

24 hours, cells were treated with 200uM $CoCl_2$ (Cobalt(II) chloride hexahydrate, Sigma-Aldrich, USA) diluted in ethanol for 4 hours.

RNA extraction and qRT-PCR

RNA extraction was performed using RNeasy Mini Kit (Qiagen, USA) and cDNA was synthesized using the Omniscript Reverse Transcription kit (Qiagen, USA), following the manufacturer's instructions. Quantitative-Real-Time-PCR (qRT-PCR) reaction was performed with TaqMan Gene Expression Assays (Applied Biosystems, USA), using gene-specific IDT probes (Integrated DNA Technologies, Inc., USA): *CDH3* (Hs.PT.51.5028751), *GLUT1* (Hs.PT.47.19044492.g), *CAIX* (Hs.PT.47.14063.g), and *GAPDH* (Hs.PT.39a.22214836). Analysis was performed with the ABI PRISM 7700 Sequence Detection System Instrument and software (Applied Biosystems, USA), following the manufacturer's recommendations. The internal standard human *GAPDH* was used to normalize cDNA quantity. Data was analyzed by the comparative 2 (-ΔΔCT) method [33]. For all data comparisons, the Student's t-Test was used (two tailed, unequal variance). All reactions were done in triplicate and the results presented as mean of the values from three or more independent experiments.

Immunofluorescence and confocal microscopic analysis

Cells were cultured on glass coverslips and 24 hours later they were treated either with 200uM of $CoCl_2$ or with the respective vehicle (Ethanol) during 4 hours. After that, cells were fixed with 4% paraformaldehyde (20 minutes), treated with NH4Cl (50 mM) for 10 minutes, washed with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes, at room temperature. Unspecific reactions were blocked with 30 minutes incubation of cells with blocking solution (5% BSA in PBS-tween 0,5%). Cells were then stained with the primary antibodies, followed by incubation in the dark of Alexa488 or Alexa-594-conjugated secondary IgG (Dako Cytomation, Carpinteria, CA) in a 1:500 dilution. Primary and secondary antibodies were diluted in blocking solution. Each sample was mounted with Vectashield (Vector Laboratories, Inc, Burlingame, CA) containing DAPI and visualized with Leica SP5 confocal microscope (Leica Microsystems GmbH, Germany). Volume of cells of both conditions was acquired by Z-stack measurements.

FACS analysis and sorting

For FACS analysis, cells were harvested with versene/0.48 mM EDTA (Invitrogen, UK), washed with PBS supplemented with 0.5% FBS and re-suspended in the stain buffer (2 mM EDTA and 0.5% bovine albumin in PBS). Single cell suspension was labeled with APC-conjugated P-cadherin, GLUT1 and CAIX antibodies. Cells transfected with the control siRNA and with *CDH3* siRNA were doubled stained either with P-cadherin and GLUT1 or CAIX antibodies. A live-dead stain (Invitrogen, UK) and the primary and secondary antibodies were incubated at 4°C, in the dark, for 15 minutes. Secondary Alexafluor-488-conjugated goat anti-rabbit IgG (Invitrogen, UK) was used in a 1:100 dilution. The labeled cells were then washed in the stain buffer and analyzed on a FACS Canto-II (BD Biosciences, USA).

For the sorting experiments, the subpopulations of SUM149 and BT20 breast cancer cells were selected according to P-cadherin expression (highest and lowest 20% expressing cells). Cells were sorted using BD Influx or FACS ARIA-II (BD Biosciences) and collected into 10% Hanks buffered solution (Invitrogen, UK). The purity of sorted populations was 80-95%. In addition, a further sample was also collected of cells passed through the laser under pressure, but not sorted, to act as a control for the effect of the pressure on the cells.

Protein extraction and western blot analysis

Protein lysates were prepared from sorted cells, using catenin lysis buffer [1% (v/v) Triton X-100 and 1% (v/v) NP-40 (Sigma-Aldrich, USA) in deionized phosphate-buffered saline (PBS)] supplemented with 1:7 proteases inhibitors cocktail (Roche Diagnostics GmbH, Germany) for 10 min, at 4°C. Cell lysates were mixed with a vortex and centrifuged at 14000 rpm at 4°C, during 10 min. Supernatants were collected and protein concentration was determined using the Bradford assay (BioRad Protein Assay kit, USA). Proteins were dissolved in sample buffer [Laemmli with 5% (v/v) 2-b-mercaptoethanol and 5% (v/v) bromophenol blue] and boiled for 5 min at 95°C or at 65° (for GLUT1 staining). Samples were separated by SDS–PAGE and proteins were transferred into nitrocellulose membranes [Amersham Hybond enhanced chemiluminescence (ECL)]. For immunostaining, membranes were blocked with 5% (w/v) non-fat dry milk in PBS containing 0.5% (v/v) Tween20 and incubated during 1 hour with anti-P-cadherin and anti-GAPDH, two hours with anti- CAIX and anti- GLUT1 and overnight with anti-HIF-1α. After washes with PBS-Tween20, membranes were incubated with HRP-conjugated anti-mouse, goat or rabbit secondary antibodies (Santa Cruz Biotechnologies, USA) diluted 1:2000 for one hour. Proteins were then detected using ECL reagent (Amersham, USA) as a substrate. Quantity One software (BioRad, USA) was used for quantification of the differences in protein expression comparing with GAPDH expression.

Mammosphere forming efficiency (MFE) assay

After the 72 h of the siRNA transfection, cells were enzymatically harvested and manually disaggregated with a 25-gauge needle to form a single-cell suspension

and resuspended in cold PBS. Cells were plated at 500/cm2 in nonadherent culture conditions, in flasks coated with 1.2% poly(2-hydroxyethylmethacrylate)/95%ethanol (Sigma-Aldrich, USA) and allowed to grow for 5 days, in DMEM/F12 containing B27 supplement, and 500 ng/ml hydrochortisone, 40 ng/ml insulin, 20 ng/ml EGF in a humidified incubator at 37°C and 5% (v/v) CO2. Mammosphere forming efficiency was calculated as the number of mammospheres (≥50 μm) formed divided by the cell number plated, being expressed as a percentage.

Statistical analysis

For immunohistochemistry results on breast cancer samples, statistical analysis was performed by SPSS statistics 17.0 software (SPSS Inc., USA). χ^2 test and contingency tables were used to determine associations between groups and the results were considered statistically significant when the p-value was lower than 0.05. Concerning the functional *in vitro* assays, all were performed independently and in triplicate. Data were analyzed using the unpaired Student *t* test and considered statistically significant when the *P* value was less than 0.05. Statistical analyses were performed using Office Excel 2010 (Microsoft Corporation, Reading, UK). All statistical tests were two-sided.

Results

P-cadherin overexpression is significantly associated with the expression of hypoxic, glycolytic and acidosis markers in primary invasive breast carcinomas

In a large series of invasive breast carcinomas, previously classified for molecular subtypes [27], immunohistochemistry staining was performed for P-cadherin, HIF-1α, GLUT1, CAIX, MCT1, MCT4, and CD147 (Figure 1). Membrane P-cadherin expression was found in 145/468 (31%) of the cases. Nuclear HIF-1α was considered positive in 104/315 (33%) carcinomas. Concerning the membrane expression of GLUT1, CAIX, MCT1, MCT4 and CD147, we observed 140/327 (42.8%), 66/316 (20.8%), 106/407 (26%), 69/419 (16.5%) and 24/217 (11%) positive cases, respectively. Membrane GLUT1 and CAIX expression was frequently detected in peri-necrotic tumor areas (Figure 1).

The association between the expression of each one of these markers with the classical breast cancer prognostic factors (Table S2 in Additional file 1) as well as with the molecular subtypes and biomarkers ER, PgR, HER2 and Ki67 (Table S3 in Additional file 1) was evaluated. As previously reported, P-cadherin expression was significantly associated with high-grade carcinomas ($p < 0.0001$), HER2-overexpressing and basal-like molecular subtypes ($p < 0.0001$), ER and PgR negativity ($p < 0.0001$), high expression of HER2 ($p < 0.0001$), as well as with high Ki67 ($p = 0.0141$). Accordingly, HIF-1α expression was also associated with grade III ($p < 0.0001$) and high proliferative ($p = 0.0197$) tumors. Concerning the expression of GLUT1, CAIX, MCT1 and CD147, all have been significantly associated with high-grade ($p < 0.001$), basal-like ($p < 0.001$), ER and PgR negative ($p < 0.05$) tumors; absence of lymph node metastasis was more frequently observed in MCT1 expressing tumors ($p = 0.0223$) and CAIX expression was associated with an increased tumor size ($p = 0.0005$). Additionally, the expression of GLUT1, MCT1 and CD147 was associated with high proliferation indexes measured by Ki67 expression ($p = 0.0339$, $p = 0.0297$, $p = 0.0179$, respectively). There was still an expected significant association between the expression of hypoxic, glycolytic and acid-resistant phenotype markers (Table S4 in Additional file 1).

Interestingly, P-cadherin overexpression was also significantly associated with the expression of HIF-1α ($p < 0.0001$), GLUT1 ($p < 0.0001$), CAIX ($p < 0.0001$), MCT1 ($p = 0.0337$) and CD147 ($p < 0.0001$) (Table S5

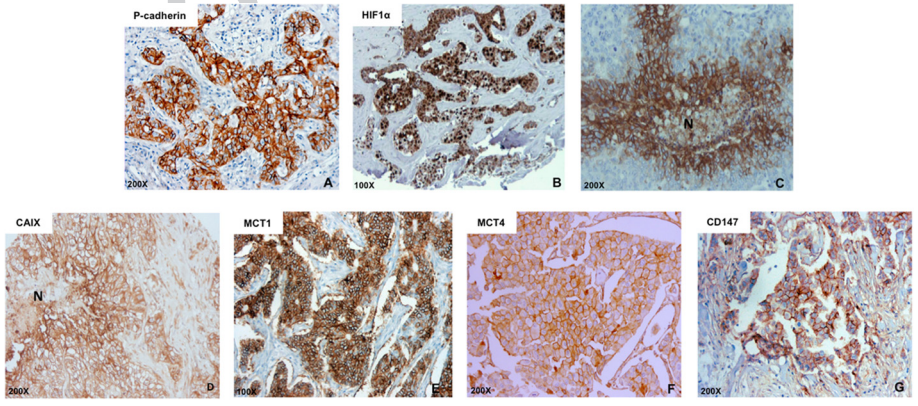

Figure 1 Immunoexpression of P-cadherin, HIF-1α, GLUT1, CAIX, MCT1, MCT4 and CD147 in breast cancer samples. Immunohistochemical staining for P-cadherin **(A)**, HIF-1α **(B)**, GLUT1 **(C)**, CAIX **(D)**, MCT1 **(E)**, MCT4 **(F)** and CD147 **(G)** expression in primary invasive breast carcinomas. Images **A**, **C**, **D**, **F** and **G** are in 200x magnification; **B** and **E** are in 100X magnification. N: necrosis.

in Additional file 1); in contrast, no association was found with MCT4 expression *(p = 0.553)*. With this data, we could demonstrate that breast carcinomas with positive expression to HIF-1α, GLUT1, CAIX, MCT1 and CD147 are significant associated to tumors showing a high percentage of cancer cells stained for the basal epithelial marker P-cadherin (Figure 2). F2

HIF-1α stabilization by $CoCl_2$ increases membrane P-cadherin expression in breast cancer cells

Due to the direct association found between P-cadherin and HIF-1α expression in invasive breast carcinomas, as well as to the described association between both markers and the maintenance of stem-like properties, we decided to evaluate if HIF-1α stabilization could be affecting the expression of P-cadherin in breast cancer cells. Furthermore, by the use of a bioinformatics prediction tool [34,35], we were able to recognize a putative binding site for HIF-1 transcription factor, positioned within a CpG island of *CDH3*/P-cadherin promoter [36]. Thus, we treated SUM149 breast cancer cells with $CoCl_2$ in order to increase the expression of HIF-1α (Figure 3A). F3 Although there were no alterations in *CDH3* mRNA levels ($p = 0.562$, Figure 3B), we could observe, by FACS analysis, a statistically significant increase of membrane P-cadherin expression upon HIF-1α stabilization ($p = 0.0246$; Figure 3C). We confirmed this result by confocal microscopy (Figure 3D), where we noticed that $CoCl_2$ treatment resulted in nuclear accumulation of HIF-1α, as well as in an increased expression of P-cadherin at the cell membrane, when compared with cells treated only with the vehicle (ethanol). Although not statistically significant ($p = 0.0716$), we also observed a decrease in the cellular height after $CoCl_2$ treatment (Figure 3E), indicating a re-organization of the cytoskeleton after HIF-1α stabilization, which can be associated with the induction of P-cadherin expression.

P-cadherin expression interferes with *GLUT1* and *CAIX* mRNA levels in breast cancer cells

The direct associations found in primary invasive breast carcinomas established, for the first time, a connection between P-cadherin expression and metabolic alterations of tumor cells. Therefore, in order to find out if these associations were reflecting a crosstalk between P-cadherin expression and the metabolic shift experienced by breast cancer cells, we decided to silence *CDH3* transcripts by siRNA-mediated knockdown in basal-like P-cadherin over-expressing breast cancer cell models (BT20 and SUM149). By real-time PCR, we could observe that *CDH3* silencing led to a statistically significant downregulation of *GLUT1* and *CAIX* mRNA in BT20 breast cancer cells ($p < 0.05$) (Figure 4A). Although not statistically significant, we could also find a tendency to a decrease in *GLUT1* and *CAIX* mRNA levels in SUM149 breast cancer cells (Figure 4D). No significant alterations were found in the mRNA expression of *HIF-1α*, *MCT1* and *CD147* upon *CDH3* silencing in both cell lines (Figure 4A and D). Interestingly, when *GLUT1* (Figure 4B and E) and *CAIX* (Figure 4C and F) were silenced, there were no significant alterations in *CDH3* mRNA levels. F4

P-cadherin is co-expressed with GLUT1 and CAIX in basal-like breast cancer cell lines

The above results led us to go further on the relationship between P-cadherin and GLUT1 and CAIX, since we observed that the expression of these both molecules was being somewhat responsive to P-cadherin in breast cancer cells. Thus, we decided to study if there was an enrichment of P-cadherin expression in GLUT1 and/or CAIX positive populations. Interestingly, we observed that cells presenting the highest expression of P-cadherin (20% high P-cad) were the ones also presenting the highest expression of GLUT1 and CAIX, while the ones

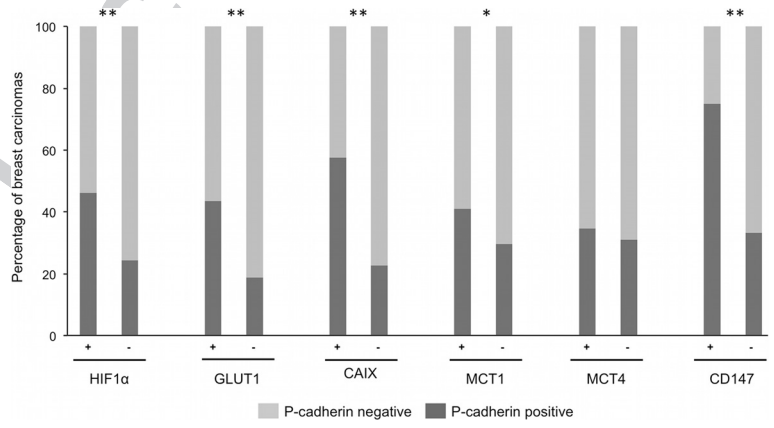

Figure 2 Aberrant P-cadherin expression in HIF-1α, GLUT1, CAIX, MCT1, MCT4 and CD147 expressing breast carcinomas. P-cadherin overexpression was significantly associated with the expression of HIF-1α, GLUT1, CAIX, MCT1 and CD147. No association was found with MCT4 expression. *$p < 0.05$; **$p < 0.0001$.

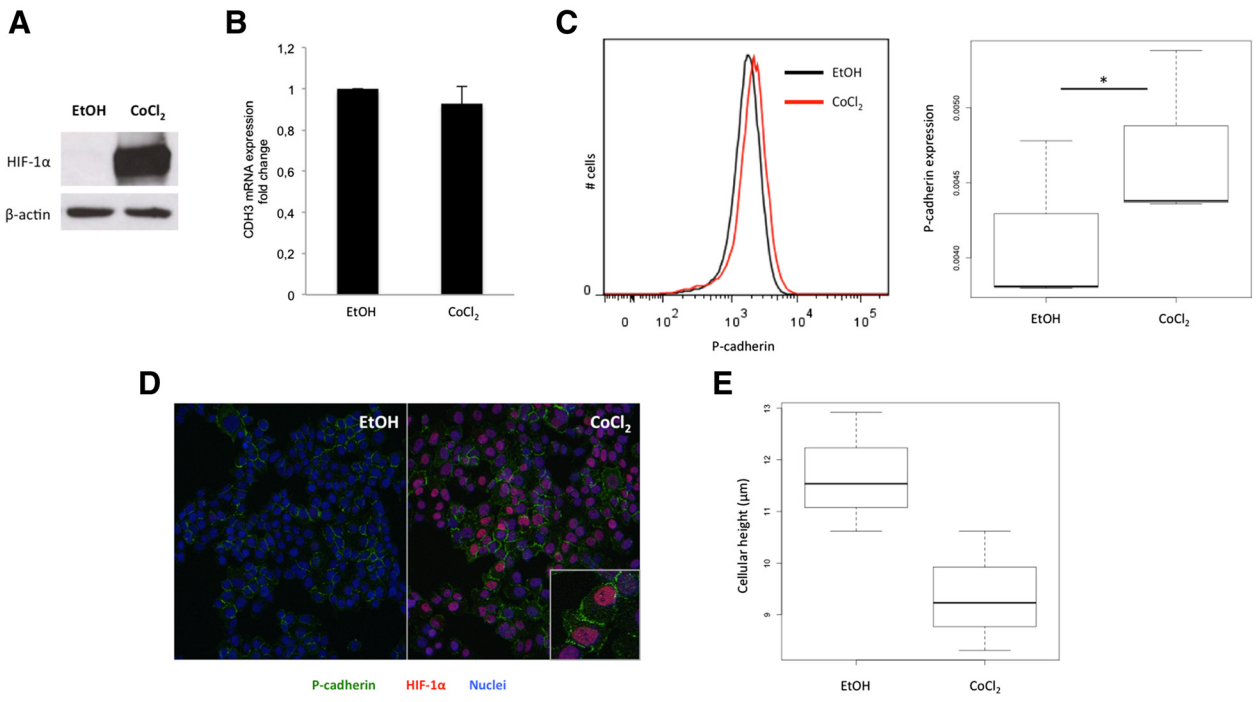

Figure 3 HIF-1α stabilization induces the expression of P-cadherin in the membrane of SUM149 breast cancer cells. HIF-1α stabilization and accumulation by $CoCl_2$ treatment was confirmed by western blot **(A)**. qRT-PCR showed no significant alterations ($p = 0.562$) in *CDH3* mRNA levels upon $CoCl_2$ treatment **(B)**. Using FACS analysis, a statistically significant increase ($p = 0.0246$) was observed in the expression of membrane P-cadherin in $CoCl_2$ treated cells, when compared with the control cells treated with the vehicle (EtOH) **(C)**. Immunofluorescence of $CoCl_2$ treated cells showed nuclear HIF-1α expression (red) and an increase of membrane P-cadherin expression (green), compared with vehicle treated cells **(D)**. Z-stack measurements revealed a decrease in the height of $CoCl_2$-treated cells comparing with the control cells **(E)**. *$p < 0.05$.

showing the lowest expression of P-cadherin (20% low P-cad) demonstrated the lowest levels of GLUT1 and CAIX (Figure 5A). This result was confirmed when we sorted and separated the 20% high and low P-cadherin cell populations and analyzed the expression of GLUT1 and CAIX by western blot (Figure 5B). Furthermore, when we selected the population of cells by their GLUT1 expression, the 20% high/low GLUT1 cells also presented the highest and lowest levels of P-cadherin expression, respectively (Figure 5C). Still, the cells selected by CAIX expression also presented the same tendency concerning P-cadherin expression (Figure 5D). Similar results were obtained in BT20 breast cancer cells (Figure S1 in Additional file 2).

The inhibition of *CDH3*, *HIF-1α*, *GLUT1* and *CAIX* affects MFE in basal-like breast cancer cells

Our previous results show that HIF-1α stabilization was accompanied by an increase of membrane P-cadherin expression, which was co-expressed with GLUT1 and CAIX in breast cancer cells. Since it has been already described that HIF-1α, GLUT1 and CAIX are required for CSC survival and tumor aggressiveness, and that we have recently shown that P-cadherin is also involved in the maintenance of stem-like properties of basal-like breast CSCs, we decided to evaluate the effect of the inhibition of all these molecules, alone or in combination, on the mammosphere forming efficiency (MFE%) of the SUM149 breast cancer cell model (Figure 6). As expected, silencing the expression of *CDH3*, as well as of *HIF-1α*, *GLUT1* and *CAIX*, showed a significant decrease of the ability to form mammospheres when compared with the cells transfected with the control siRNA ($p = 0.0153$, $p = 0.0156$, $p = 0.000284$ and $p = 0.000902$, respectively). Moreover, when we simultaneously silenced *CDH3* and *HIF-1α*, we also observed a decrease of MFE of the target cells ($p = 0.0367$), although not cumulative. If, in addition to *CDH3* and *HIF-1α*, we silence the downstream targets *GLUT1* and *CAIX* (siRNA *CDH3* + *HIF-1α* + *GLUT1* + *CAIX*), there is still a non-cumulative decrease effect in MFE (%) ($p = 0.0152$).

Discussion

Much attention has been paid to the tissue microenvironment, highlighting the importance of hypoxia as a mediator of cell survival, pluripotency, stemness and proliferation of

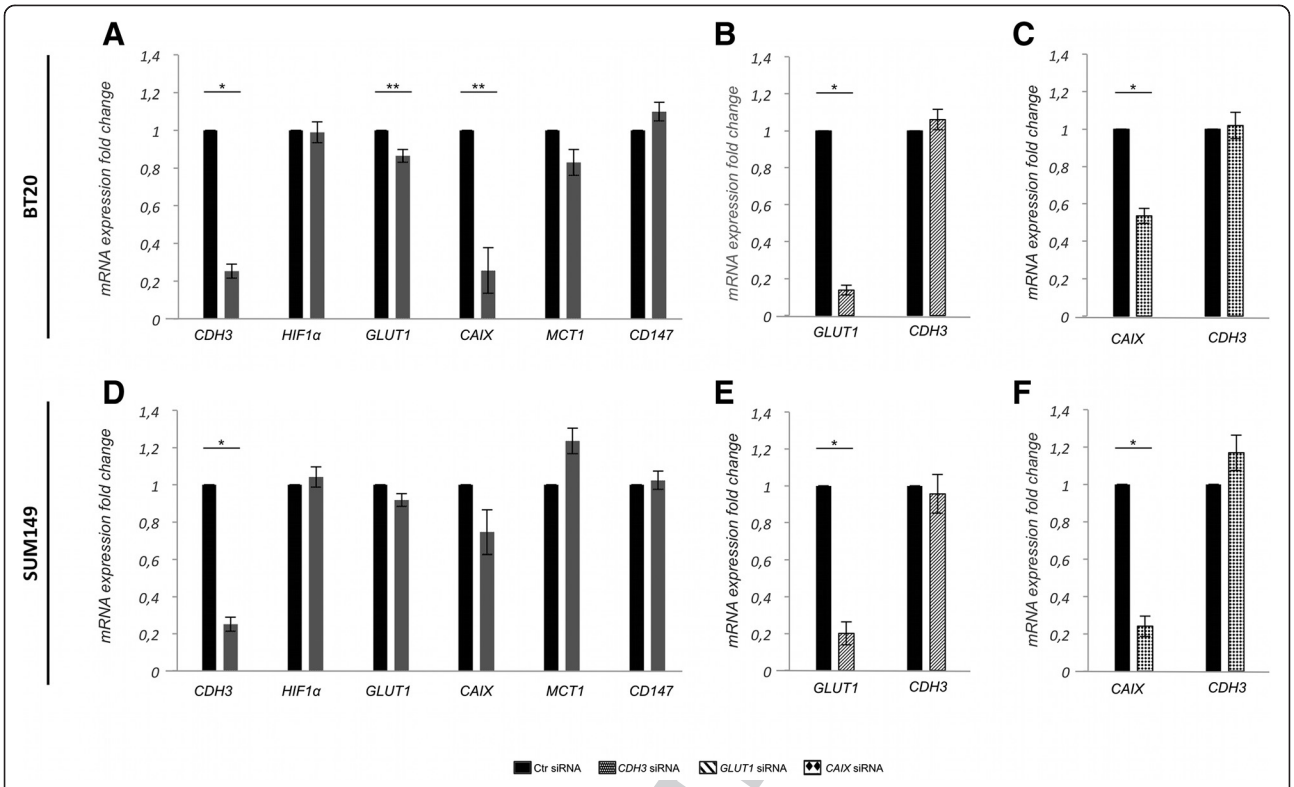

Figure 4 P-cadherin expression affects *GLUT1* and *CAIX* mRNA levels in breast cancer cells. mRNA expression measured by qRT-PCR of *CDH3*, *HIF-1a*, *GLUT1*, *CAIX*, *MCT1 and CD147* when inducing siRNA-mediated knockdown of *CDH3*, *GLUT1* and *CAIX* in BT20 and SUM149 breast cancer cell lines. Upon *CDH3* silencing, there was a significant decrease of the mRNA expression of *GLUT1* and *CAIX* in BT20 **(A)** and a tendency, although not statistically significant, to a decrease in SUM149 cells **(D)**. No differences were observed in the mRNA expression of *HIF-1a*, *MCT1* and *CD147* in both cell lines. On the other hand, there were no alterations in *CDH3* mRNA expression in BT20 **(B and C)** and in SUM149 **(E and F)** breast cancer cell lines, when *GLUT1* **(B and E)** and *CAIX* **(C and F)** were silenced. *$p < 0.05$; **$p < 0.0001$.

normal stem cells [2,3] Alterations in metabolism have also been proven to be important for the maintenance of stem cell properties [3]. hESC and iPSC tends be more glycolytic and less oxidative comparing to their somatic counterparts [37-39]. Interestingly, the behavior of some tumor cells resembles in many aspects the behavior of hESC, as well as adult stem cells [3].

P-cadherin has been recently described as a mediator of stem-like properties in basal-like breast cancer cell lines. The main goal of this work was to understand if there was an association between P-cadherin overexpression and the phenotype of adaptation of breast cancer cells to microenvironment stresses, such as hypoxia, lactic acidosis and glycolytic metabolism, also characteristics of the undifferentiated state of stem, progenitor and breast CSC. Interestingly, we showed that P-cadherin is associated to the hypoxic phenotype and metabolic reprogramming of breast cancer cells.

We demonstrated an association between the expression of P-cadherin and HIF-1α in breast carcinomas. Similarly to P-cadherin, HIF-1α expression is associated to worse prognosis in breast cancer, short patient's survival, high proliferation and poor tumor differentiation [7,40]. Interestingly, we found that HIF-1α stabilization promotes membrane P-cadherin expression in breast cancer cells, although no alterations were detected in *CDH3* mRNA levels after $CoCl_2$ treatment. With these results, we can hypothesize that increased P-cadherin membrane expression observed after HIF-1α accumulation occurs at a post-transcriptional level, and not as a direct effect. Thus, based on our findings and on previous data reported by others, the crosstalk between these molecules might be explained by several hypotheses. Since hypoxia, through HIF-1α [41], is able to induce ER degradation and that ER is a transcription *CDH3* repressor [26,42], it is possible that hypoxia can be inducing HIF-1α, promoting ER degradation and allowing *CDH3* transcription. Other possible explanation is related to the link between hypoxia and BRCA1, another *CDH3* transcriptional repressor [43]. It is known that hypoxia induces BRCA1 downregulation [44] and that *BRCA1*-mutated breast carcinomas, which are enriched in HIF-1α expression [45], present aberrant expression of P-cadherin [46].

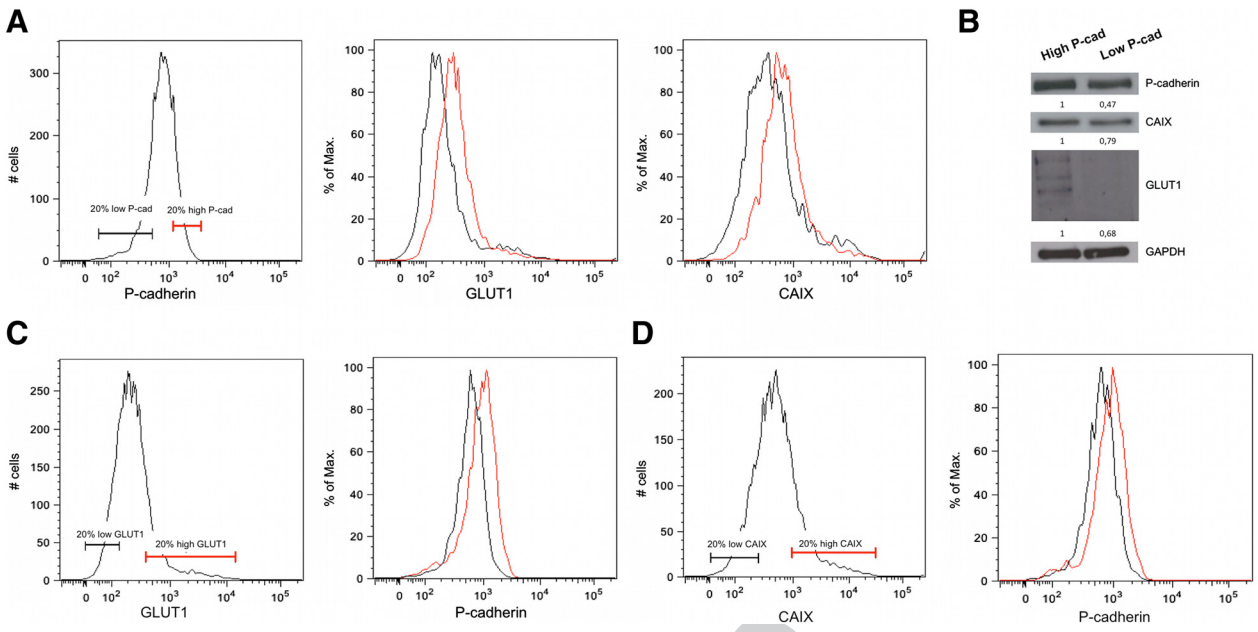

Figure 5 P-cadherin is co-expressed with GLUT1 and CAIX in basal-like breast cancer cell lines. By flow cytometry analysis, we observed that the 20% of cells with the highest and lowest P-cadherin expression presented highest and lowest, respectively, expression of GLUT1 and CAIX **(A)**. When cells were sorted by P-cadherin expression, lysed and analyzed in SDS-PAGE, the same result was observed concerning the high and low expression of GLUT1 and CAIX **(B)**. On the other hand, 20% of cells with the highest and lowest levels of GLUT1 and CAIX (**C** and **D**, respectively) expression also presented highest and lowest P-cadherin expression.

Concomitantly with the increase of membrane P-cadherin expression, we still observed a decrease in the cellular height after $CoCl_2$ stabilization, indicating a putative re-organization of the cytoskeleton and cell morphology, with the acquisition of a phenotype associated to breast cancer cell aggressiveness [47]. This observation is consistent with our previous data, showing that P-cadherin expression is able to induce invasion, migration and motility in breast cancer cells [28] and that its modulation also interferes with GTPase-mediated signal transduction and actin cytoskeleton organization [29].

Our results also showed an association between P-cadherin and GLUT1, CAIX, MCT1 and CD147. These molecules are important response mediators of cancer cell survival in stressful microenvironments and are upregulated in poor prognosis BLBC [12,13], where P-cadherin is aberrantly expressed. Upregulation of membrane transporters, such as CAIX and MCTs, is responsible for the extracellular acidification, assisting *in vitro* cancer cell invasion and *in vivo* metastization through the acidic degradation of the ECM [48]. CD147/EMMPRIN is known to induce MMP production [49], such as MMP2 [50], to promote tumor growth, inhibit cell apoptosis and enhance cell migration under hypoxic conditions [51]. Interestingly, the highly significant association found between P-cadherin and CD147 is in accordance with the above-described reports and also with our previous data, showing that P-cadherin induces an invasive behavior in breast cancer cells through the activation of MMP1 and MMP2 [28].

Using basal-like breast cancer cell lines, we found that P-cadherin silencing is able to induce the downregulation of *GLUT1* and *CAIX* mRNA, whereas *GLUT1* and *CAIX* knockdown showed little or no effect in *CDH3* mRNA expression. Thus, although the expression variations found were not so prominent as expected for direct molecular targets, we believe that P-cadherin is putatively involved in a signaling pathway that interferes with the metabolic reprograming of cancer cells. Moreover, we also demonstrated that the cell subpopulation expressing more/less P-cadherin at the cell surface was the same presenting higher/lower levels of GLUT1 and CAIX and vice-versa. These results suggests that P-cadherin overexpressing breast cancer cells are most likely to exhibit increased glycolysis and to survive to metabolic-driven pH alterations, justifying the enhanced aggressive behavior and metastatic properties.

In breast cancer, hypoxia and HIF-1α have a pivot role in promoting tumor growth and metastasis through the maintenance, expansion as well as increased activity of breast CSCs [52-54]. Oliveira-Costa *et al.* showed that HIF-1α was differently expressed in $CD44^{+}/CD24^{-/low}$ breast cancer cells [55]. Moreover, CAIX also plays a role in the regulation of stemness and expansion of breast CSCs in hypoxic niches [56]. Recently, our group demonstrated that P-cadherin expression is able to mediate stem cell

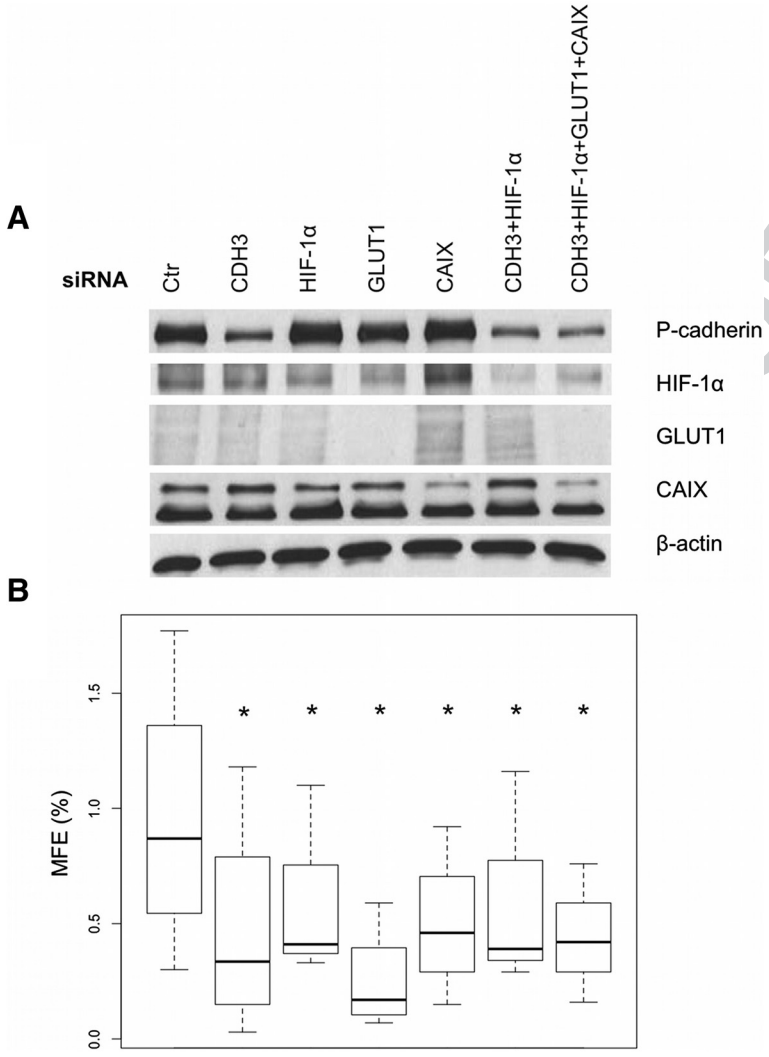

Figure 6 MFE (%) decreases upon siRNA-mediated silencing of *CDH3, HIF-1α, GLUT1* and *CAIX* in SUM149 cells. siRNA-mediated silencing of *CDH3, HIF-1α, GLUT1* and *CAIX* was confirmed by western blot analysis **(A)**. A statistically significant decrease in MFE (%) was observed when we silenced *CDH3, HIF-1α, GLUT1* and *CAIX*. The simultaneous silencing of *CDH3* and *HIF-1α* also revealed a significant decrease of MFE (%) of the target cells, although not cumulative. Still, the silencing of the expression of all transcripts tested led to a non-cumulative inhibition of the MFE compared to the control cells **(B)**. *$p < 0.05$.

properties in basal-like breast cancer cells [30]. Still, we showed that BLBC, which are enriched in P-cadherin expression, present a high percentage of cells with CSC phenotype [57]. Accordingly, we demonstrated that the inhibition of *CDH3, HIF-1α, GLUT1* and *CAIX* in basal-like breast cancer cells significantly reduces their MFE, an important measure of breast cancer stem cell activity. We still show that the silencing of *CDH3* and *HIF-1α,* or even the simultaneous inhibition of *CDH3, HIF-1α, GLUT1* and *CAIX,* have a non-cumulative effect in the inhibition in MFE comparing to the single silencing of any of these molecules. This result is in accordance with our observation that the cell subpopulation expressing increased levels of P-cadherin also presents higher levels of GLUT1 and CAIX, demonstrating that we are targeting the same breast cancer stem cell population.

Taking together, we believe that the hypoxic environment in breast tumors might be selecting a pool of cancer cells with stem-like properties, showing increased P-cadherin expression as well as a distinct metabolic state. These characteristics will be accounted for therapeutic resistance, since breast CSCs are resistant to radiotherapy and are thought to be responsible for breast cancer metastasis [32,58]. Interestingly, previous data from our group also demonstrated that P-cadherin inhibition sensitizes breast cancer cells to radiation-induced cell death [30].

Conclusions

In conclusion, we demonstrated that aberrant P-cadherin expression is associated with the hypoxic/glycolytic and acid-resistant phenotype in invasive breast carcinomas, represented by a panel of markers including HIF-1α, GLUT1, CAIX, MCT1 and CD147. We also showed that membrane P-cadherin expression can be increased by HIF-1α stabilization, as well as can modulate *GLUT1* and *CAIX* expression. Moreover, we still demonstrated that P-cadherin is differentially expressed in basal-like breast cancer cells that also present higher levels of GLUT1 and CAIX. Thus, we believe that HIF-1α might be stabilizing membrane P-cadherin expression in breast CSC or even selecting P-cadherin-high expressing breast CSCs in the hypoxic stem cell niche. In its turn, P-cadherin expression is probably shifting the metabolic program of these cells, which might be responsible for tumor aggressiveness, as well as for their ability to survive, compared to the low P-cadherin expressing cells. In this case, P-cadherin is mediating the survival of aggressive cells thought to be resistant to standard cancer therapies, being responsible for tumor relapses and metastasis in breast cancer patients.

Additional files

Additional file 1: Table S1. Clinical, pathological and immunohistochemical characteristics of the 473 primary invasive breast carcinomas*. **Table S2.** Association of P-cadherin, HIF-1α, GLUT1, CAIX, MCT1, MCT4 and CD147 and classic prognostic factors in breast cancer*. **Table S3.** Association of biomarkers and classical molecular markers used in breast cancer*. **Table S4.** Association between the hypoxia, glycolytic and acid-resistant phenotype markers within the series of invasive breast carcinomas*. **Table S5.** P-cadherin overexpression is associated with the expression of markers of hypoxia, glycolytic and acid-resistant phenotype in breast cancer*.

Additional file 2: Figure S1. P-cadherin is co-expressed with GLUT1 and CAIX in BT20 cell line.

Abbreviations

BLBC: Basal-like breast carcinomas; CAIX: Carbonic anhydrase IX; CSC: Cancer stem cells; EMMPRIN: Extracellular matrix metalloproteinase inducer; ER: Estrogen receptor; GLUT1: Glucose transporter 1; hESC: Human embryonic stem cells; iPSC: Induced-pluripotent stem cells; MCT: Monocarboxylate transporter; MFE: Mammosphere forming efficiency; MMPs: Metalloproteinases; PgR: Progesterone receptor; ROS: Reactive oxygen species; TEBs: Terminal end buds; TICs: Tumor-initiating cells; TMA: Tissue microarrays.

Competing interests

The authors declare that they have no financial or non-financial competing interests.

Authors' contributions

BS was involved in study design, carried out the majority of the experimental work and drafted the manuscript. ASR, ARN and AFV helped with practical experimentation in molecular and cell biology; NL, DM, CP and AA were involved in the construction and characterization of the breast cancer series used in the study. FS and RG were the pathologists that evaluated the immunohistochemical reactions. Also, JP, FS and FB provided assistance in data analysis and interpretation. Finally, JP conceived the study and participated in manuscript production. All authors were involved in writing the paper and had final approval of the submitted and published versions.

Acknowledgements

We thank to Dr. Luiz Zeferino, Dra. Rozany Dufloth, Dra. Daniella Vieira, Dr. Vitor Carneiro and Dr. Luiz Veronese for providing the breast cancer samples from the Department of Pathology of General Hospital of UNIMED (Araçatuba, São Paulo, Brazil), Federal University of Santa Catarina (Florianopolis, Brazil), Department of Pathology of Hospital of Divino Espírito Santo (Ponta Delgada, Portugal), and from Veronese Patologia e Citologia Araçatuba Pathology Laboratory. (Araçatuba, São Paulo, Brazil). We also thank Patricia Oliveira for the help in statistical and bioinformatics tools. Furthermore, we thank to Catarina Leitão from IBMC (Institute for Molecular and Cell Biology) the help in FACS experiments and analysis. Finally, we thank the Bioimaging Center for Biomaterials and Regenerative Therapies (b.IMAGE) for help with confocal microscopy.
This work was funded by FEDER funds through the COMPETE Program (Programa Operacional Factores de Competitividade) and by national funds through FCT (Portuguese Foundation for Science and Technology, Portugal), mainly in the context of the scientific project PTDC/SAU-GMG/120049/2010-FCOMP-01-0124-FEDER-021209, and partially by PTDC/SAU-FCF/104347/2008. FCT funded the research grants of BS (SFRH/BD/69353/2010), ASR (SFRH/BPD/75705/2011), ARN (grant from the project PTDC/SAU-GMG/120049/2010), CP (SFRH/BPD/ 69479/2010), AV (SFRH/BPD/90303/2012), as well as JP, with Programa Ciência 2007 (Contratação de Doutorados para o SCTN – financiamento pelo POPH – QREN – Tipologia 4.2 – Promoção do Emprego Científico, comparticipado pelo Fundo Social Europeu e por fundos nacionais do MCTES) and Programa IFCT (FCT Investigator). IPATIMUP is an Associate Laboratory of the Portuguese Ministry of Science, Technology and Higher Education and is partially supported by FCT.

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Received: 18 June 2014 Accepted: 18 September 2014
Published: 1 October 2014

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doi:10.1186/1471-2407-14-734
Cite this article as: Sousa *et al.*: The basal epithelial marker P-cadherin associates with breast cancer cell populations harboring a glycolytic and acid-resistant phenotype. *BMC Cancer* 2014 **14**:734.

PUBLICATIONS

Paper 2

Int. J. Dev. Biol. 55: 811-822
doi: 10.1387/ijdb.113382aa

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

P-cadherin role in normal breast development and cancer

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ABSTRACT P-cadherin is a cell-cell adhesion molecule, whose expression is highly associated with undifferentiated cells in normal adult epithelial tissues, as well as with poorly differentiated carcinomas. Its expression has been already reported in human embryonic stem cells and it is presumed to be a marker of stem or progenitor cells of some epithelial tissues. In normal breast, P-cadherin has an essential role during ductal mammary branching, being expressed by the monolayer of epithelial cap cells at the end buds. In mature mammary tissue, its expression is restricted to the myoepithelium; it has been postulated that it may also be present in early luminal progenitor cells. In breast cancer, P-cadherin is frequently overexpressed in high-grade tumours, being a well-established indicator of poor patient prognosis. It has been reported as an important inducer of cancer cell migration and invasion, with underlying molecular mechanisms involving the signalling mediated by its juxtamembrane domain, the secretion of matrix metalloproteases to the extracellular media, and the cleavage of a P-cadherin soluble form with pro-invasive activity. Intracellularly, this protein interferes with the endogenous cadherin/catenin complex, inducing p120-catenin delocalization to the cytoplasm, and the consequent activation of Rac1/Cdc42 and associated alterations in the actin cytoskeleton. Considering P-cadherin's role in cancer cell invasion and metastasis formation, a humanized monoclonal antibody was recently produced to antagonize P-cadherin-associated signalling pathways, which is currently under Phase I clinical trials. In this review, the most important findings about the role of P-cadherin in normal breast development and cancer will be illustrated and discussed, with emphasis on the most recent data.

KEY WORDS: *P-cadherin, CDH3 gene, mammary gland, breast cancer*

Introduction

Classical cadherins constitute a family of molecules that mediate calcium-dependent cell-cell adhesion, localized to the adherens-type junctions. The intracellular domains of cadherins bind directly to cytoplasmic catenins, which link them with the actin cytoskeleton, providing the molecular basis for stable cell interactions. The cadherin/catenin complex, as well as the signalling pathways controlled by this structure, represent a major regulatory mechanism that guide cell fate decisions, through its influence on cell growth, differentiation, motility, and survival (Cavallaro and Dejana, 2011).

Classical cadherins include *CDH1*/E-cadherin (epithelial), *CDH2*/N-cadherin (neuronal), *CDH3*/P-cadherin (placental) and *CDH4*/R-cadherin (retinal), designated by their tissue distribution. E-cadherin is the predominant cadherin family member expressed in all epithelial tissues, being extremely important to the maintenance of the cell shape and polarity; in fact, it is well known that *CDH1* acts as a tumour suppressor gene, negatively regulating the invasion and metastasis of tumour cells in several malignancies (Yilmaz and Christofori, 2010). In contrast, N-cadherin is up-regulated in several cancers and contributes to an invasive phenotype by interacting with fibroblast growth factor receptor (FGFR) and its

Abbreviations used in this paper: α–ctn, α-catenin; βctn, β-catenin; CBD, catenin-binding domain; CDH, cadherin; C/EBPβ, CCAAT/enhancer-binding protein β; CK, cytokeratin; CSC, cancer stem cell; CTC, circulating tumour cell; E-cad, E-cadherin; EC, epithelial cell; EEM, ectodermal dysplasia, ectrodactyly, and macular dystrophy; EGFR, epidermal growth factor receptor; ER, estrogen receptor; EMT, epithelial-to-mesenchymal transition; FGFR, fibroblast growth factor receptor; HDAC, histone deacetylase; HJMD, hypotrichosis with juvenile macular dystrophy; IBC, inflammatory breast cancer; JMD, juxtamembrane domain; MECs, myoepithelial cells; MFE, mammosphere forming efficiency; NAF, nipple aspirate fluid; P-cad, P-cadherin; PgR, progesterone receptor; p120ctn, p120-catenin; SHFM, split hand/foot malformation; sP-cad, soluble P-cadherin; TEB, terminal end buds; TSA, trichostatin A.

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Final, author-corrected PDF published online: 29 November 2011

ISSN: Online 1696-3547, Print 0214-6282

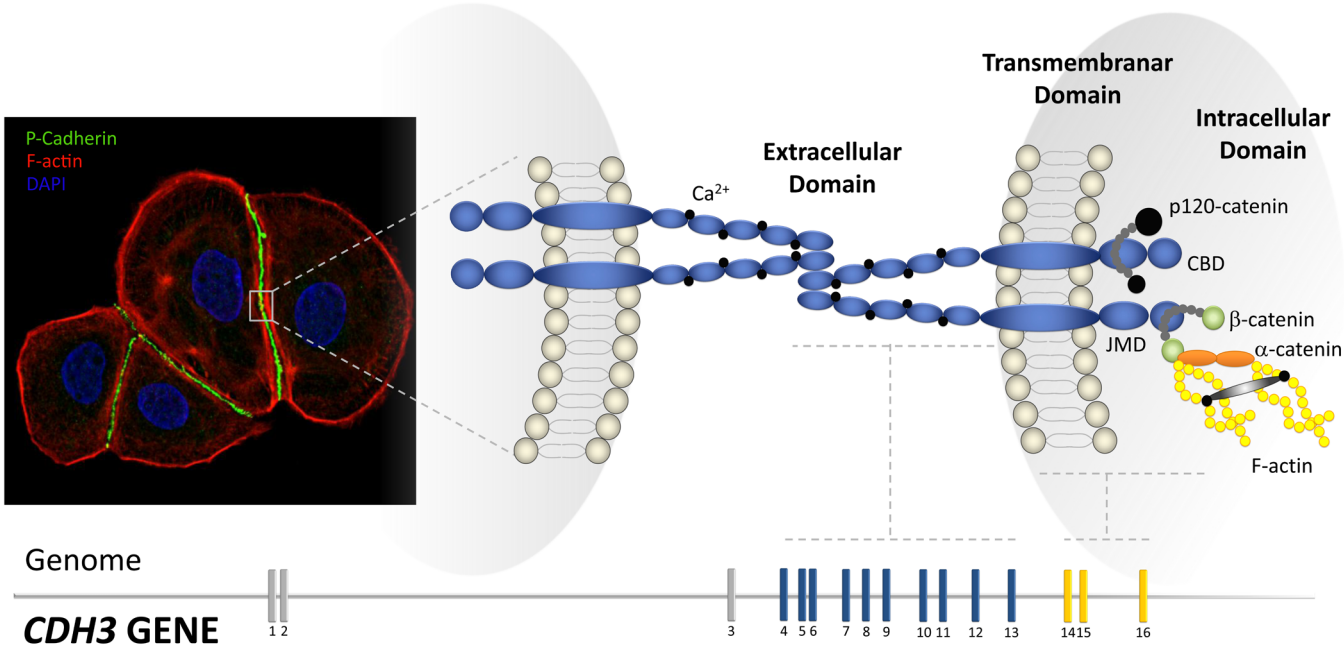

Fig. 1. Schematic representation of the structural components of the P-cadherin adhesive junction. *Lateral clustering of P-cadherin molecules is required to form stable cell-to-cell contacts in BT-20 breast cancer cells [immunofluorescence: P-cadherin (green), F-actin (red), DAPI (blue)]. In the intercellular space, P-cadherin extracellular domains interact with P-cadherin extracellular domains of adjacent cells to mediate cell adhesion. The intracellular catenins bind to the cytoplasmic tail of P-cadherin. p120-catenin binds the cadherin tail at the juxtamembrane domain (JMD), whereas* β*-catenin binds to the distal catenin binding domain (CBD).* α*-catenin associates with* β*-catenin and is directly linked to the actin cytoskeleton. The lower panel shows the genomic structure of* CDH3*/P-cadherin gene, which is constituted by 16 exons: the extracellular part of P-cadherin is encoded by 10 exons (exons 4-13), whereas the transmembrane and intracellular domains are determined only by the information included in the last 3 exons (exons 14-16).*

downstream signalling (Suyama *et al.*, 2002).

P-cadherin is also often reported to correlate with increased tumour cell motility and invasiveness when overexpressed (Cheung *et al.*, 2010, Paredes *et al.*, 2004, Ribeiro *et al.*, 2010, Taniuchi *et al.*, 2005). Although the role of P-cadherin encoding gene (*CDH3*) in cancer is far less well characterized than the one attributed to *CDH1*, the opposite effects in mammary cancer are weird, since these molecules share more than 67% of homology (Hulpiau and van Roy, 2009). The *CDH3* gene harbours 16 exons (Fig. 1) and maps to chromosome 16q22.1, a region that contains a cluster of several cadherin genes, just 32 kilobases upstream of the gene encoding human E-cadherin (Bussemakers *et al.*, 1994). The mature P-cadherin glycoprotein structure is similar to that of classical cadherins, comprising three distinct domains (extracellular, transmembrane and intracellular), in order to promote homotypic interactions. At the cell membrane, these create lateral dimers that act together in a zipper-like structure between neighbouring cells (Shapiro *et al.*, 1995) (Fig. 1).

The function and strength of P-cadherin-mediated adhesion depends on its dynamic association with catenins, which link the cadherin cytoplasmic tail to the actin cytoskeleton and facilitate clustering into the junctional structure, forming cadherin/catenin complexes. This tail comprises two main domains: the juxtamembrane domain (JMD), which has been suggested to play a critical role in cadherins stability at the cell membrane, and the catenin-binding domain (CBD), which is known to be essential for cadherin function. The p120-catenin (p120ctn), β-catenin (βctn) and α-catenin (αctn) are the major documented interaction partners that bind to cadherin intracellular domains and allow the binding to the actin cytoskeleton of the cell (Green *et al.*, 2010) (Fig. 1).

P-cadherin upregulation was frequently observed in various malignant tumours, including breast, gastric, endometrial, colorectal and pancreatic carcinomas, and is correlated with poor survival of breast cancer patients (Hardy *et al.*, 2002, Imai *et al.*, 2008, Paredes *et al.*, 2005, Stefansson *et al.*, 2004, Taniuchi *et al.*, 2005). In contrast, significantly low levels of the P-cadherin gene expression were detected in a diverse panel of normal tissues (Imai *et al.*, 2008). Thus, disruption of P-cadherin signalling represents an intriguing opportunity for the development of novel targeted therapeutic agents in cancer.

P-cadherin role in epithelial cell differentiation

Classical cadherins play important roles in maintaining the structural integrity of epithelial tissues and are mainly involved in cell differentiation during embryogenesis. There are several indications in the literature that point to the relationship between cell adhesion molecules and stem cell features, not only as biomarkers that help to isolate and characterise stem cells, but also as important mediators of stem cell activity, via modulation of signalling pathways (Raymond *et al.*, 2009). Regarding the classical cadherins, an important amount of data comes from the identification of P-cadherin as a marker of undifferentiated stem or progenitor cells (Kendrick *et al.*, 2008, Raymond *et al.*, 2009).

In a very recent study, it has been shown that *CDH3* is one of the genes that encode a surface protein that identify the pluripotent population of human embryonic stem cells (Kolle *et al.*, 2009). This expression is concomitant with the one of E-cadherin, which was

shown to be present even at the one cell stage of embryogenesis (Hyafil *et al.*, 1980) (Fig. 2A). In fact, mouse embryo implantation into the uterine epithelium involves both E- and P-cadherin. The most dramatic expression of P-cadherin was observed in the placenta, both in the embryonic and maternal regions, hence the classical denomination of placental-cadherin. The expression of P-cadherin in the uterus began with the appearance of the decidua, into which the extraembryonic cells expressing P-cadherin of implanted embryos invade to establish the embryo-maternal connection (Aplin *et al.*, 2009, Nose and Takeichi, 1986). Early reports specified low expression in human placenta (Shimoyama *et al.*, 1989), although P-cadherin is detectable where trophoblasts adjoin (cytotrophoblast-cytotrophoblast and cytotrophoblast–syncytiotrophoblast) in the first trimester villus, with some immunoreactivity still detectable at term (Aplin *et al.*, 2009) (Fig. 2A).

In contrast, E-cadherin was found expressed only in the embryonic region of placenta with a sharp boundary to the maternal region. These observations may suggest complementary roles of the two cadherins, such that P-cadherin is required for association of embryonic and maternal tissues during the late implantation stage, while E-cadherin is essential in preventing the embryonic tissues from mixing with the maternal tissues (Aplin *et al.*, 2009, Nose and Takeichi, 1986) (Fig. 2A).

It was also shown that E- and P-cadherins are both expressed in the ectoplacental cone, ectoderm, some endodermal tissues and nephric tubules, whereas both P- and N-cadherins are expressed in each cell of the lateral plate mesoderm, corneal endothelium, and pigmented retina (Nose and Takeichi, 1986) (Fig. 2A).

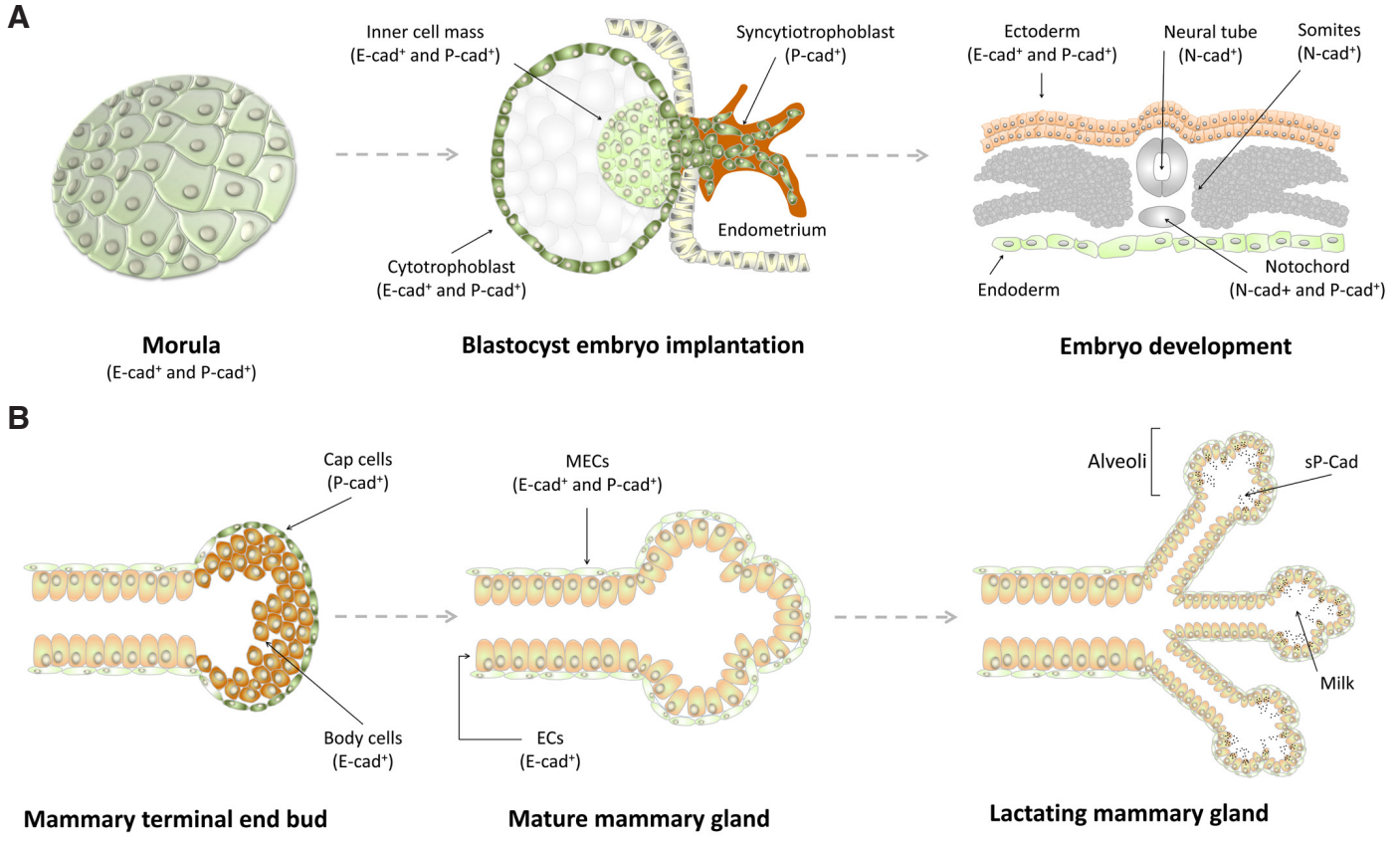

Fig. 2. Schematic representation of P-cadherin expression during embryogenesis and mammary gland development. (A) *Undifferentiated embryonic stem cells included in the morula, as well as in the inner mass of the blastocyst express E- and P-cadherin. During the blastocyst embryo implantation in the endometrial lining of the uterus, the trophectoderm differentiates into the cytotrophoblast and syncytiotrophoblast, which are key steps in placental development. There is an E-cadherin downregulation in the syncytiotrophoblast, which mainly expresses P-cadherin, while cells actively invade the uterine wall. Early in embryonic development, there is the formation of the neural tube, where a strip of specialized cells, forming the notochord, induces the cells of the ectoderm directly above it to become the primitive nervous system. Meanwhile, the ectoderm and endoderm continue to curve around and fuse beneath the embryo to create the body cavity, completing the transformation of the embryo from a flattened disk to a three–dimensional body. It is known that the ectoderm is E- and P-cadherin positive, which will give rise to the skin and its appendages. After cadherin switch, the neural tube becomes N-cadherin positive, as well as the somites. It was described that the notochord is N- and P-cadherin positive.* **(B)** *The ducts of the developing mammary gland are established, with their inner luminal epithelial cell layers (ECs) and outer myoepithelial cell layers (MECs), while the terminal end buds (TEB) move through the mammary fat pad. It is thought that cap cells at the tip of the TEB, which are P-cadherin positive, generate transit cells of the myoepithelial lineage on the outer side of the TEB (E- and P-cadehrin positive); at the same time, these cells also generate transit cells that form the central TEB mass, known as body cells, which will constitute the luminal epithelial lineage (E-cadherin positive). The ductal lumen is formed as body cells enter in apoptose and outer cells differentiate into luminal epithelial cells. Extracellular-matrix enzymes degrade the stroma in front of the TEB to enable it to move through the fat pad; however, it is unclear how the structures actually move through the gland. During lactation, secretory cells in the breast alveoli become P-cadherin positive at the cytoplasm, and secrete a soluble form of this protein (sP-cad) that is found in the milk.*

In adult tissues, the expression of P-cadherin is mainly found in the basal layer of several epithelial structures, such as skin, uterine cervix, prostate, and lung, contributing to the maintenance of the epithelial phenotype. The expression of cadherin molecules was extensively studied in mouse epidermis, in adulthood and during fetal development, where has been found that E-cadherin is expressed both in the basal and intermediate layers of epidermis, whereas P-cadherin is only expressed in the basal and proliferative layer (Pizarro *et al.*, 1995). Furthermore, loss of E-cadherin plays an important role in bud formation and in the acquisition of an invasive behaviour, whereas P-cadherin becomes predominant expressed later in development, namely in the growing hair follicle and in the early progenitor cells from hair germs and small hair placodes (Fujita *et al.*, 1992, Rhee *et al.*, 2006). Like hair follicles, sweat glands and mammary glands develop also from the same discrete accumulation of stem cells resting in the primitive epidermis, the outermost cell layer of an embryo, and there is strong evidence that dynamic changes in the composition of adherens junctions are important for the development of skin appendages (Fujita *et al.*, 1992).

The final evidence showing the importance of P-cadherin for the architecture and development of epithelial tissues was demonstrated by human genetic syndromes that are induced due to P-cadherin loss. *CDH3* gene mutations have been shown to cause P-cadherin functional inactivation, leading to developmental defects associated with two inherited diseases in humans: 1) hypotrichosis with juvenile macular dystrophy (HJMD) and 2) ectodermal dysplasia, ectrodactyly, and macular dystrophy (EEM syndrome). The common features of both diseases are sparse hair and macular dystrophy of the retina, while only EEM syndrome shows the additional finding of split hand/foot malformation (SHFM) (Kjaer *et al.*, 2005, Sprecher *et al.*, 2001). No defects were described for these conditions, concerning the human mammary development, or other epithelial bud structures. However, it is known that during bud patterning, a special arrangement occurs, where cells change their interaction with their neighbours and break their attachments to the extracellular matrix (ECM). Cells achieve this by activating specific transcriptional programs (Shimomura *et al.*, 2008).

P-cadherin role in normal breast development

Two members of the cadherin family are found to be expressed in the normal adult mature non-lactating mammary gland, usually at sites of cell-to-cell contact: E-cadherin is present in both luminal epithelial (ECs) and myoepithelial cells (MECs), whereas P-cadherin is confined to the myoepithelium (Paredes *et al.*, 2002). This type of cell localization is already found during mammary gland development, since P-cadherin expression is only found in the precursor cells of the myoepithelial compartment, the cap cells of the ductal end buds, whereas luminal cells and body cells do not show any expression of P-cadherin and are typically E-cadherin positive (Daniel *et al.*, 1995) (Fig. 2B).

Besides the restricted expression of P-cadherin in the normal breast, this protein is extremely important to the establishment of the correct architecture of the tissue, as demonstrated by functional-blocking antibody experiments *in vitro* and *in vivo*. Daniel and collaborators exposed the end buds and mature mammary glands of 5 week-old virgin mice to slow-release plastic implants liberating a monoclonal antibody for P-cadherin. No effect in the luminal layer was found, but disruption of the basally located cap cell layer was clearly observed (Daniel *et al.*, 1995). Also, more recently, Chanson *et al.,* described that P-cadherin contributes specifically to the organization of the myoepithelial cell layer of the breast, since when an antibody that blocks P-cadherin function was used in an *in vitro* self-organizing assay of the human mammary bilayer, the migration of MECs, occurring during normal sorting of both layers, was compromised (Chanson *et al.*, 2011). These experiments indicate that selective expression of P-cadherin in the basal layer is necessary for the maintenance of mammary tissue integrity.

In fact, deletion of P-cadherin affects normal mammopoiesis, since the *CDH3*-null female mice exhibit precocious mammary gland differentiation in the virgin state, and breast hyperplasia and dysplasia with age (Radice *et al.*, 1997). These observations in knockout animals indicate P-cadherin cell-cell interactions and signalling as regulatory determinants of the negative growth of the luminal epithelium, being important for the maintenance of an undifferentiated state of the normal mammary gland.

Interestingly, the expression of this adhesion molecule is activated in human mammary luminal cells during late pregnancy and lactation (Soler *et al.*, 2002). However, in these alveolar lactating cells, P-cadherin expression pattern is not restricted to the cell-cell borders, but shows a cytoplasmic staining, typical of a secreted protein. Indeed, in human milk, a soluble fragment of P-cadherin (sP-cad) with 80KDa was found to be present, corresponding to the extracellular domain of the molecule (Soler *et al.*, 2002) (Fig. 2B). Recently, Mannello and collaborators showed that the highest concentration of sP-cad is detected in milk collected during the first trimester of lactation (Mannello *et al.*, 2008). Still, it is not clear which is the biological and physiological role attributed to this fragment in the normal function of the breast. Some authors suggest a role for sP-cad in alveolar differentiation during lactation, or in the immune response of the mother or the baby, or as a signalling protein between epithelial and myoepithelial cells. Further studies are in progress to determine the sites of proteolysis of the sP-cad-secreted protein in different body fluids where it has been previously described (such as milk, serum, semen, nipple aspirate fluid (NAF), breast cyst fluid and amniotic fluid) (Mannello *et al.*, 2008, Soler *et al.*, 2002).

Prognostic relevance of P-cadherin in breast cancer

As mentioned above, P-cadherin is expressed in normal breast MECs and in MECs associated with non-invasive breast proliferations, showing no significant cross-reactivity with luminal/ECs, stromal myofibroblasts and blood vessels (Reis-Filho *et al.*, 2003). However, P-cadherin was described as being overexpressed in 20% to 40% of invasive breast carcinomas, as well as in 25% of ductal carcinomas *in situ* (DCIS) (Paredes *et al.*, 2007a, Paredes *et al.*, 2007b, Paredes *et al.*, 2002). Most important, several studies have reported P-cadherin as a marker of poor prognosis in breast cancer, since P-cadherin-positive carcinomas were significantly associated with short-term overall and disease-specific survival, as well as with distant and loco-regional relapse-free interval (Gamallo *et al.*, 2001, Paredes *et al.*, 2005, Peralta Soler *et al.*, 1999, Turashvili *et al.*, 2011).

P-cadherin expression has also been positively associated with high histological grade tumours, as well as with well-established

markers of poor prognosis, like Ki-67, epidermal growth factor receptor (EGFR), cytokeratin 5 (CK5), vimentin, p53, and HER2 expression and negatively associated with age at diagnosis, hormonal receptors (ER and PgR), and Bcl-2 expression. Interestingly, none of these reports showed a significant association with tumour size and lymph node metastasis (Gamallo *et al.*, 2001, Paredes *et al.*, 2005, Peralta Soler *et al.*, 1999, Turashvili *et al.*, 2011).

Besides the strong association between P-cadherin expression, poor patient prognosis and tumour aggressiveness, transgenic mice overexpressing *CDH3*/P-cadherin in the luminal epithelial layer of the mammary gland, under the control of the MMTV promoter, showed normal morphogenesis, architecture, lactation and involution, and no mammary tumours formed spontaneously (Radice *et al.*, 2003). Nevertheless, Mannello *et al.,* demonstrated a significant increased shedding of sP-cad in NAFs from women with breast cancer when compared with healthy subjects or with women with pre-cancer conditions, suggesting its possible release via proteolytic processing in cancer cells (Mannello *et al.*, 2008).

P-cadherin: marker of histological and molecular subtypes in breast cancer

Besides the strong association between P-cadherin expression and poor patient prognosis, no significant correlation was ever observed between this protein and a specific breast cancer histological type. The majority of positive P-cadherin tumours are invasive ductal carcinomas NOS, or carcinomas with metaplastic or medullary features (Paredes *et al.*, 2005, Reis-Filho *et al.*, 2003, Turashvili *et al.*, 2011). The observation that metaplastic and medullary breast carcinomas are consistently immunoreactive for P-cadherin supports a myoepithelial/basal transcriptomic programme for these lesions (Han *et al.*, 1999, Jacquemier *et al.*, 2005). Han and coworkers reported P-cadherin expression in almost all studied cases of medullary, carcinosarcomas, and sarcomatoid metaplastic breast carcinomas (Han *et al.*, 1999); in addition, all the metaplastic cases that we have studied were positive for at least one basal/myoepithelial marker, including P-cadherin (Reis-Filho *et al.*, 2003). We also showed that P-cadherin expression, in canine malignant tumours, was significantly related to spindle cell carcinoma, carcinosarcoma and osteosarcoma. In these lesions, both carcinomatous and sarcomatous components of carcinosarcoma expressed P-cadherin (Gama *et al.*, 2004, Gama *et al.*, 2008).

Concerning molecular profiling classification, at least five subtypes of invasive breast carcinoma were identified (Luminal A and B, Normal-like, HER2-overexpressing and Basal-like), exhibiting distinct clinical prognostic behaviour (Perou *et al.*, 2000). P-cadherin is one of the most important biomarkers to identify basal-like and HER2-overexpressing breast cancers (Arnes *et al.*, 2005, Paredes *et al.*, 2007b, Turashvili *et al.*, 2011). Basal-like breast cancer expresses genes characteristic of basal epithelial cells, which include, besides P-cadherin, high-molecular weight basal cytokeratins (CK5/6, CK14, CK17), vimentin, αB-crystalline, caveolins1/2 and EGFR (Arnes *et al.*, 2005). Until now, the most accepted criterion to identify basal-like breast carcinomas, by immunohistochemistry, is the triple negative phenotype along with CK5 and/or EGFR positivity (Nielsen *et al.*, 2004). However, we demonstrated that P-cadherin expression shows higher sensitivity to distinguish the basal phenotype of breast carcinomas, being a reliable option compared to the "gold standard" pair CK5/EGFR (Sousa *et al.*, 2010). Although this still need validation by gene expression profiles, these results can introduce the idea of using P-cadherin as an additional option in the daily workup of breast pathology laboratories to identify basal-like breast cancers.

P-cadherin is also prominently expressed in inflammatory breast cancer (IBC), which is a distinct and aggressive form of locally-advanced breast cancer, with high metastatic potential and high death rate. These tumours are characterized by frequent basal and HER2 phenotypes but, surprisingly, luminal IBC also express the basal marker P-cadherin (Ben Hamida *et al.*, 2008). This profile suggests a specificity that needs to be further investigated.

Interestingly, the expression profiling of *BRCA1*-deficient hereditary tumours has identified a pattern of gene expression similar to basal-like breast tumours (Palacios *et al.*, 2003). Very recently, Gorski *et al.* showed that *BRCA1* and *c-Myc* form a repressor complex on the promoters of specific basal genes, including *CDH3* gene, and represent a potential mechanism to explain the observed overexpression of key basal markers in *BRCA1*-deficient tumours (Gorski *et al.*, 2010). Actually, it has been shown that P-cadherin expression in breast carcinomas is strongly associated with the presence of *BRCA1* mutations (Arnes *et al.*, 2005).

P-cadherin role in adhesion, invasion and motility

Carcinomas progress by promotion of local invasion and distant metastasis. The acquisition of this invasive behaviour is one of the first steps in the metastatic process. Those cancer cells often develop alterations in their shape, as well as in their attachment to other cells and to ECM. Therefore, cell-cell and cell-matrix interactions play the most important role during tumour progression, since disruption of cell-cell adhesion during carcinogenesis is the basis for motility, invasion and metastasis of tumour cells (Yilmaz and Christofori, 2010).

P-cadherin has been detected as altered in various human tumours, but its effective role in the carcinogenesis process remains discussible, since it behaves differently depending on the studied tumour cell model and context. For instance, in a colorectal cancer cell line (HT-29), P-cadherin has been suggested to act as a pro-adhesive and anti-invasive/anti-migration molecule, exactly as E-cadherin (Van Marck *et al.*, 2011). Also, in melanomas, P-cadherin behaves as an invasion suppressor gene. Indeed, in highly invasive melanoma cell lines (that lack E-cadherin expression), P-cadherin overexpression was able to promote the formation of cell-cell contacts and counteract invasion (Van Marck *et al.*, 2005). The anti-invasive effect of P-cadherin was also recently confirmed in *in vivo* experiments, showing that its expression is refractory to invasive signals induced by myofibroblasts. Nevertheless, it was found a secreted truncated variant of P-cadherin in malignant melanomas, which negatively regulates cell-cell adhesion and induces a more motile phenotype, thus playing an important role in migration and metastasis of melanoma cells (Bauer and Bosserhoff, 2006).

On the other hand, in several other models, including breast cancer, P-cadherin behaves as an oncogene, and is often reported to correlate with increased tumour cell motility and invasiveness when aberrantly expressed (Cheung *et al.*, 2010, Mandeville *et al.*, 2008, Paredes *et al.*, 2007a, Paredes *et al.*, 2004, Taniuchi *et al.*, 2005, Van Marck *et al.*, 2011). Using *in vitro* breast cancer cell models, we found that overexpression of P-cadherin promotes single cell motility, directional cell migration, as well as invasion

capacity through the matrigel matrix (Ribeiro *et al.*, 2010). This same migratory phenotype was observed in bladder, pancreatic and cholangiocarcinoma cancer cell lines (Baek *et al.*, 2010, Mandeville *et al.*, 2008, Taniuchi *et al.*, 2005, Van Marck *et al.*, 2011).

Curiously, we have noticed that P-cadherin is able to induce invasion only in cell systems which already express an endogenous and functional cadherin, like E-cadherin in breast cancer cells, or N-cadherin in HEK293T cells and PDAC pancreatic cancer cells (Paredes *et al.*, 2004, Ribeiro *et al.*, 2010, Taniuchi *et al.*, 2005). Based on this hypothesis, we have recently proved that P-cadherin is able to interact with E-cadherin in breast tumours and cancer cells, promoting cancer cell invasion by disrupting the interaction between E-cadherin and both p120ctn and βctn. In the absence of E-cadherin expression, in the same cancer model, P-cadherin is able to suppress invasion by its strong interaction with catenins, surrogating the role of E-cadherin in cell-cell adhesion (unpublished data).

P-cadherin role in EMT and cadherin switch

Among the cadherin families, E-cadherin and N-cadherin are the most highly characterized subgroup of adhesion proteins. E-cadherin is ubiquitously expressed throughout most epithelial tissues and serves as a negative regulator to functionally block the βctn signalling pathway and suppress tumour cell growth and invasion (Onder *et al.*, 2008). However, numerous preclinical and clinical studies have shown that the loss of E-cadherin occurs concurrently with the upregulation of N-cadherin or other cadherin family members implicated in invasive growth, like P-cadherin or cadherin-11. This process, known as cadherin switching, has been reported to promote epithelial-to-mesenchymal transition (EMT) and leads to tumour cell invasion and metastasis (Thiery *et al.*, 2009).

Indeed, the switch from E- to N-cadherin is the one better known and reported by several studies. N-cadherin overexpression, via cadherin switching, was observed in various invasive cancer cell lines and tumours, namely from the esophagus, prostate, cervix, and ovary. This specific cadherin switch leads to the inhibition of cell-cell contacts and elicits active signals that support tumour-cell migration, invasion, and metastatic dissemination (Thiery *et al.*, 2009).

The cadherin switch from E- to P-cadherin is a common event during embryo development; however, few reports describe it during tumour progression. Indeed, some invasive and aggressive epithelial tumours, namely the local advanced IBC, and some highly metastatic breast cancer cells, as the 4T1 cell model, maintain E-cadherin expression at the cell membrane and show aberrant concomitant expression of P-cadherin (Ben Hamida *et al.*, 2008, Lou *et al.*, 2008). Nevertheless, there are some reports showing a switch from these two epithelial cadherins during tumour progression, namely in ovarian, endometrial and bladder carcinoma (Bryan *et al.*, 2008, Patel *et al.*, 2003, Stefansson *et al.*, 2004). In all these studies, P-cadherin increased expression significantly correlated with decreased E-cadherin expression and, consequently, represented a key step in disease progression. However, it has been already shown that, in cholangiocarcinoma cells, the E- to P-cadherin switch does not induce EMT signalling, since does not affect the expression of mesenchymal markers, such as Snail 1 and 2, vimentin, and fibronectin (Baek *et al.*, 2010).

Recognized regulators of *CDH3*/P-cadherin transcription

Signalling pathways or other cellular mechanisms that are involved in the regulation of cadherin-mediated adhesion are thought to underlie the dynamics of the adhesive interactions between cells.

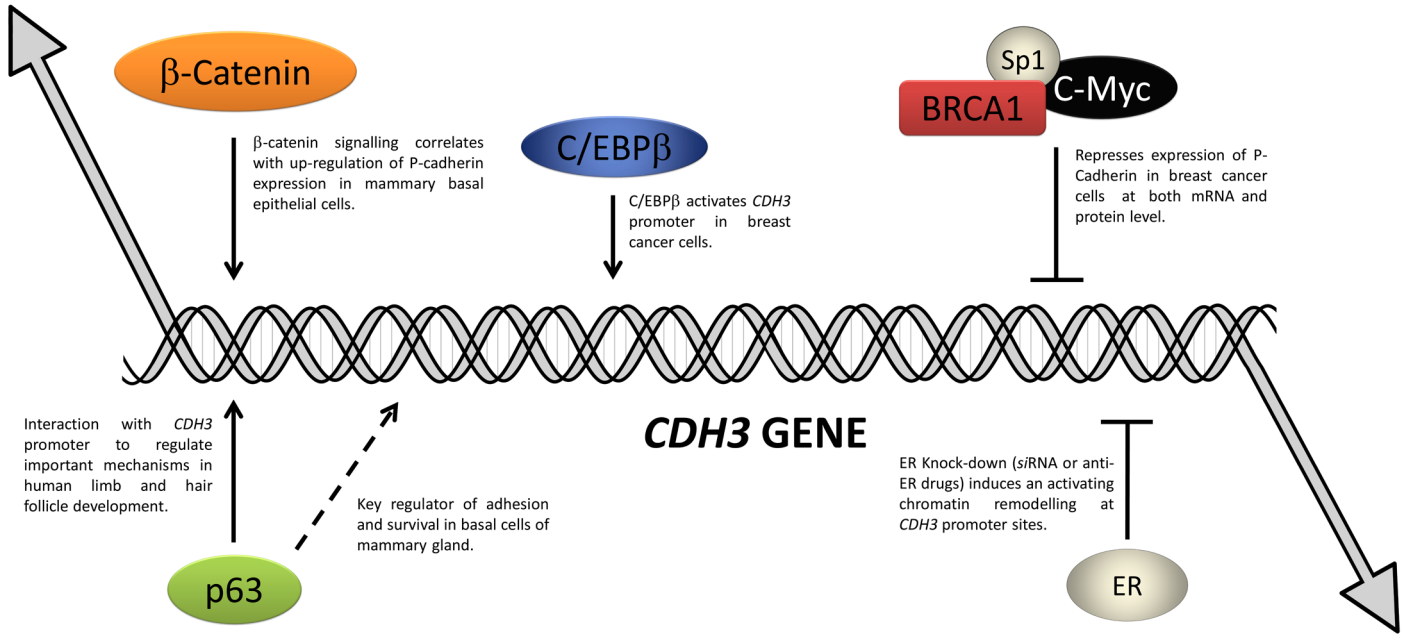

Fig. 3. Schematic representation of the described transcriptional regulators of *CDH3* /P-cadherin promoter gene. *It has been shown that β-catenin, p63 and C/EBPβ are transcriptional activators of* CDH3 *promoter, inducing its expression at the mRNA and protein level. In contrast, estrogen receptor (ER), as well as the BRCA1/c-Myc/Sp1 complex, act as transcriptional repressors of* CDH3 *promoter gene.*

Although the evidence that the expression of cadherins can result from growth factors and from changes in the promoter regions of cadherins, data concerning *CDH3* promoter regulation is still very limited.

One of the most prominent demonstrations regarding the importance of a classical transcription factor in the regulation of cell adhesion programmes in epithelial cells was demonstrated by Carroll and collaborators. This study implicated p63, a p53-family related transcription factor, as a key regulator of adhesion and survival in basal cells of the mammary gland. Importantly, the authors showed that p63 expression caused downregulation of cell adhesion-associated genes and detachment between mammary epithelial cells (Carroll *et al.*, 2006). This involvement of p63 in cell adhesion mechanisms was finally linked with *CDH3* gene, when Shimomura and colleagues demonstrated that P-cadherin is a direct p63 transcriptional target and that this interplay has a crucial role in human limb bud and hair follicle development (Shimomura *et al.*, 2008) (Fig. 3).

Furthermore, it has been shown that βctn is also associated with *CDH3* promoter activation and P-cadherin expression in basal mammary epithelial cells. Down-regulation of endogenous βctn levels inhibited *CDH3* promoter activity, while activation of βctn signalling was correlated with up-regulation of P-cadherin expression in *in vivo* mammary gland mice models, eventually contributing to the establishment of the basal phenotype (Faraldo *et al.*, 2007) (Fig. 3).

Recently, we still found that the CCAAT/enhancer-binding protein β (C/EBPβ) transcription factor was able to activate *CDH3* promoter in breast cancer cells. We showed that this novel activator of *CDH3* promoter activity exerts its activation preferably through its truncated LIP isoform, being the abundance of Sp1 sites within *CDH3* promoter a feature which potentiate the C/EBPβ-LIP activation role on *CDH3* gene (Albergaria *et al.*, 2010) (Fig. 3).

Regulation of *CDH3* gene has been also explored in terms of its transcriptional repression. In 2004, our group explored the link between ER-signalling and the regulation of P-cadherin expression in breast cancer cell lines, since we have already observed that breast tumours positive for P-cadherin expression were essentially ER negative. We verified that P-cadherin expression was induced by the pure anti-oestrogen ICI 182,780 and counteracted by 17β-oestradiol. In fact, breast cancer cells treated with ICI 182,780 showed a significant increase of P-cadherin mRNA and protein levels in a time and dose dependent manner, establishing that the lack of ER-signalling is responsible for the increase of P-cadherin, therefore, categorizing *CDH3* as an ER-repressed gene (Paredes *et al.*, 2004) (Fig. 3). Very recently, in order to deeply explore this antiestrogen-mediated mechanism, we described a cellular adaptation process where ICI 182,780 is able to induce a chromatin structural remodelling, which lead to activation of *CDH3* gene and overexpression of P-cadherin protein (Albergaria *et al.*, 2010). Such genomic de-repression effect may contribute to an augmented invasive phenotype of ER-positive breast cancer cells.

As a gene associated with the basal-like phenotype in breast cancer, *CDH3*/P-cadherin gene was recently described to be transcriptionally repressed by functional BRCA1 protein in breast cancer cell lines, at both mRNA and protein level. This same study also showed that, together with BRCA1, c-Myc form a repressor complex on the *CDH3* promoter (Fig. 3), suggesting a potential mechanism to explain the observed overexpression of key basal markers in BRCA1-deficient tumours (Gorski *et al.*, 2010).

Epigenetic regulation of P-cadherin expression

Epigenetic regulation of *CDH3*/P-cadherin has been highly reported in the last few years, with greater emphasis in cancer models. The epigenetic deregulation of P-cadherin was firstly demonstrated by Sato *et al.*, which identified *CDH3* gene promoter to be aberrantly methylated in 20% of pancreatic cancers, but not in normal pancreatic epithelia (Sato *et al.*, 2003). Similarly, *CDH3* gene was also shown to be silenced by methylation in melanoma cells (Tsutsumida *et al.*, 2004).

However, in 2005, we analysed P-cadherin promoter methylation in normal breast tissue, from which only epithelial cells were microdissected, and methylation of *CDH3* gene promoter was found in the normal epithelial/luminal cell layer from all the specimens analysed, which was associated with negative P-cadherin expression in these cells. But, in contrast to what has been verified in E-cadherin control of expression by hypermethylation of its promoter in cancer, our group found a significant correlation between P-cadherin overexpression and *CDH3* promoter hypomethylation. Using a large series of invasive breast carcinomas, we found that 71% of P-cadherin-negative breast cancer cases were methylated for the *CDH3* gene, whereas 65% of P-cadherin-positive cases were unmethylated (Paredes *et al.*, 2005).

Indeed, the genomic structure of the proximal *CDH3* gene promoter, such as the enrichment in CpG islands, as well as the attributed DNA hypersensitive sites, suggests that it is likely to be regulated by epigenetic events, others than only methylation. In fact, we observed an up-regulation of *CDH3* promoter activity and P-cadherin protein expression in cells treated with the histone deacetylases (HDAC) inhibitor Trichostatin A (TSA), showing that chromatin-activating modifications are also important in the modulation of this gene (Albergaria *et al.*, 2010). Thus, if we previously described that overexpression of P-cadherin could result from a loss of promoter methylation, we have now evidences to assume that chromatin remodelling also play an important modulator role in *CDH3* gene activity.

Reinforcing our results, *CDH3* promoter was also found hypomethylated in colonic aberrant crypt foci, in colorectal cancer, and, occasionally, in the normal epithelium adjacent to cancer (Milicic *et al.*, 2008). This hypomethylation pattern was associated with the induction of P-cadherin expression in the neoplastic colon. Finally, demethylation of the *CDH3* gene was recently detected in a large percentage of primary gastric carcinomas and was significantly associated with increasing TNM stage, suggesting that it is also a frequent event in gastric carcinomas (Kim *et al.*, 2010).

P-cadherin-downstream signalling pathways

Increasing evidences indicate that cadherins role in carcinogenesis and tumour progression do not solely lie on their adhesive function, but also depend on their interaction with other molecules (such as cytoskeletal components, integrins, and growth-factor receptors, among others) and signalling pathways (Onder *et al.*, 2008). Therefore, the stabilization of the cadherin/catenin complex represents a major regulatory mechanism for oncogenic signalling pathways, that guide cell fate decisions through the modulation of specific genes at the transcriptional level and, as a consequence, regulation of several crucial cellular processes, as proliferation, survival, polarization, differentiation, shape and migration, which

in turn affect embryogenesis, tissue formation and pathogenic events, such as cancer.

Although E-cadherin-induced signalling pathways have been extensively studied in cancer, little is known about the role of P-cadherin (Paredes *et al.*, 2004, Taniuchi *et al.*, 2005, Van Marck *et al.*, 2005). It is some kind expected that P-cadherin share common signalling pathways with other cadherins, due to its function as a cell-cell adhesion molecule; however, it is not known whether the pathways are triggered in the same way.

Sarrió and collaborators analysed microarray gene expression of a breast cancer cell line (MDA-MB-231), negative for cadherins, after expression of E- and P-cadherin. The data revealed that these molecules can activate signalling pathways leading to significant changes in gene expression. Although the expression patterns induced by E- and P-cadherin showed more similarities than differences, 40 genes were differentially modified by the expression of either cadherin type. According to data bases, these genes belonged to a wide range of biological functions, including signal transduction and growth factors (VEGFC, FGFR4), cell cycle (CCNA2), cell adhesion and ECM (CDH4, COL12A1), or cytokines and inflammation (IL24), among others (Sarrio *et al.*, 2009). This indicates that, in addition to their role in cell adhesion, E-cadherin and P-cadherin have a significant impact on the overall genetic program of breast cancer cells.

One of the molecules that have been several times referred has having a specific role in signalling related to P-cadherin is p120ctn (Fig. 4). We demonstrated that the pro-invasive activity of P-cadherin requires the JMD of its cytoplasmic tail. Transfection of HEK293T cells with several mutants of P-cadherin showed that only the ones with altered JMD were not able to induce cell invasion in *in vitro* cell models (Paredes *et al.*, 2004). Moreover, we observed that breast carcinomas co-expressing E- and P-cadherin were associated with p120ctn cytoplasmic localisation and poor patient survival (Paredes *et al.*, 2008). Since then, several other reports have been exploring that pathway. Indeed, Taniuchi *et al.,* showed that the induced cell migration by P-cadherin expression was due to activation of the Rho

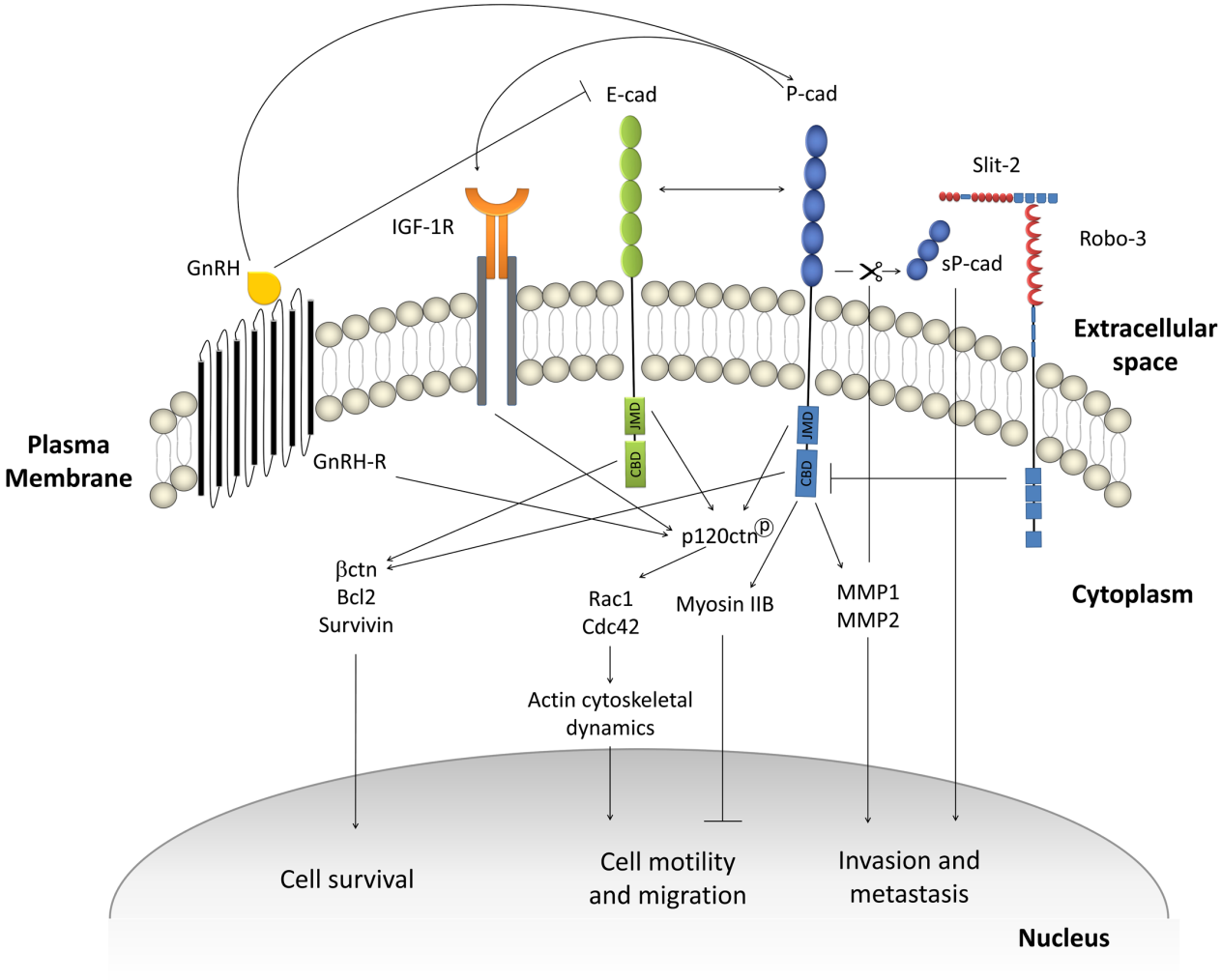

Fig. 4. Schematic representation of the signalling pathways regulated by P-cadherin expression. *P-cadherin signals are transduced by many intracellular signalling pathways, which ultimately result in alterations of the cancer cells survival, as well as cell migration and invasion capacity. For simplicity, only some of the known interactions are depicted. It should be noted that the effect of P-cadherin on the overall gene expression program of cancer cells is highly dependent on the cellular type and the biological context.*

GTPases, Rac1 and Cdc42, through accumulation of p120ctn in the cytoplasm in pancreatic cancer cell model (Taniuchi *et al.*, 2005) (Fig. 4). Very recently, P-cadherin has been also shown to cooperate with insulin-like growth factor-1 receptor to promote metastatic signalling of gonadotropin-releasing hormone in ovarian cancer via p120ctn (Cheung *et al.*, 2010). These same authors had previously shown that this p120ctn signalling mediated by P-cadherin expression, also lead to increased activity levels of Rac1 and Cdc42 (Fig. 4). Still another study has shown that p120ctn and P-cadherin, but not E-cadherin, regulate cell motility and invasion of DU145 prostate cancer cells (Kumper and Ridley, 2010).

Although binding of proteins to the JMD of P-cadherin has just been documented for p120ctn (Reynolds *et al.*, 1996), other molecules, like Hakai and presenilin-1 (PS-1), have been reported to bind to the JMD of classical cadherins. This binding is established through a sequence adjacent to, or overlapping, the p120ctn-binding domain, thereby competing with p120ctn (Baki *et al.*, 2001, Fujita *et al.*, 2002). Although the significance of these interactions is not well known, we cannot exclude the possibility that disruption of the p120ctn–binding sequence may introduce conformational changes and/or uncouples the interaction of these or other proteins, which could explain our observations. Striking examples of this were shown for E-cadherin, where functional differences have been noted between larger and minimal deletions of the JMD, with even the minimal changes disrupting binding of multiple molecules (Baki *et al.*, 2001).

Recently, it has been shown that the P-cadherin regulatory role in cell migration is also related with the expression of the non-muscle myosin II-B isoform, which is an ATP-dependent molecular motor protein that can interact with and contract filamentous actin (F-actin) (Jacobs *et al.*, 2010) (Fig. 4). These results implicate that there is a coordinated cross-talk between adhesion molecules and cellular migration-related proteins.

More recently, the role of P-cadherin was investigated in oral squamous cancer cell model, where the authors used a cell line that was deficient for classical cadherins. After P-cadherin overexpression, cells gained an epithelial-like morphology, with Snail translocation to the cytoplasm. Analysing the signalling mechanism behind it, they found glycogen-synthase-kinase-3β (GSK-3β) bound to Snail, as well as an increase in activated GSK-3β that phosphorylated Snail leading to its cytoplasmic translocation (Bauer *et al.*, 2009). These same authors also showed that Slit-2, a secreted ECM glycoprotein that acts as a molecular guidance cue in cellular migration, facilitates the interaction of P-cadherin with Robo-3, its receptor, and inhibits cell migration in oral squamous cell carcinoma cell line models (Bauer *et al.*, 2011) (Fig. 4).

In terms of breast cancer cell invasion, we found that the presence of P-cadherin, in an E-cadherin positive cellular background, is able to provoke the secretion of pro-invasive factors, such as MMP-1 and MMP-2, leading to P-cadherin ectodomain cleavage (sP-cad) which induces a pro-invasive activity by itself (Ribeiro *et al.*, 2010). This study clarified the mechanism associated to P-cadherin-induced cancer cell invasion.

Different signalling pathways should be triggered in different cell models, in order to identify new interaction partners of P-cadherin, as well as to study whether the interaction of known partner molecules differ between cadherins. Finally, it is important to highlight that the effect of cadherins on the overall gene expression program of cancer cells is highly dependent on the cellular type and the biological context. Thus, P-cadherin regulation of specific transcriptional factors may depend on the activation of other signalling pathways, or on the presence of additional molecular alterations.

P-cadherin as a breast cancer stem cell marker

An increasing body of evidence supports the notion that cancers are propagated by a small population of cells present in the malignant tissue, that possess the ability to form a hierarchy similar to the one present in normal tissues (Visvader, 2011). These cancer stem cells (CSCs) are able to proliferate, originating more stem-like cells, to exhibit resistance to current therapies and to remain quiescent during long periods of time. However, it is still not clear whether the CSC originates from the normal stem cells of the tissue that deregulate their self-renewal ability, or from normal mature cells or progenitor cells that acquired stem cell characteristics (Visvader, 2011). Importantly, attempts have been made in order to find a universal phenotype for the breast cancer stem cell; but due to the high heterogeneity of this malignancy, it is not expected that a single CSC phenotype would apply to all breast cancers.

The identification of a cancer stem cell marker for basal-like subtype of breast cancer is of particular importance, due to its high mortality rate, fast relapses and lack of target therapy (Rakha *et al.*, 2009). Recently, it has been demonstrated that the luminal progenitor of normal breast hierarchy is the cell of origin for this malignancy, since the induction of a *BRCA1* mutation in this cell was able to induce the formation of a tumour with basal phenotype (Lim *et al.*, 2009, Molyneux *et al.*, 2010). Since *CDH3* gene is repressed by BRCA1, it is likely that P-cadherin could be a good cancer stem cell marker of this specific type of tumours. In fact, using a series of breast cancer cell lines, we found that P-cadherin enriched populations (by genetic manipulation or by sorting) were enriched for mammosphere forming efficiency (MFE), as well as for the expression of CD24, CD44 and CD49f, already described as CSC markers. When compared with luminal cell lines, basal-like cell lines also showed a greater ALDEFLUOR^{bright} subpopulation and the P-cadherin positive subfraction of these cell lines was enriched in stem cell activity (MFE and 3D growth) (unpublished data). This observation linked P-cadherin expression with the luminal progenitor phenotype, which is CD44^{+}CD24^{+}CD49f^{+} (Lim *et al.*, 2009). Importantly, it has been described that the phenotype CD44^{+}CD24^{+} is tumorigenic (Meyer *et al.*, 2009). Hence, the strategy of directing therapies to the luminal progenitor phenotype, by specifically targeting P-cadherin, could potentially help to eradicate the CSCs. Interestingly, P-cadherin also conferred resistance to X-ray induced DNA damage, supporting a role for this molecule in the maintenance of yet another CSC property (unpublished data).

P-cadherin - potential therapeutic target in cancer

As clearly stated in this review, P-cadherin–mediated adhesion and the associated signalling pathways play diverse roles in the regulation of cancer cell survival, invasiveness and metastatic potential. Interestingly, in 2008, Imai and collaborators have suggested *CDH3*/P-cadherin as a possible target for immunotherapy of pancreatic, gastric, and colorectal cancers, since it was identified as a novel tumour-associated antigen, meaning that was strongly expressed in tumour cells, but not in normal cells (Imai *et al.*, 2008). Indeed, we have found that P-cadherin silencing, in breast cancer

cells inoculated in nude mice, was able to significantly inhibit *in vivo* tumour growth (unpublished data).

Recently, a novel and highly selective human monoclonal antibody against P-cadherin (PF-03732010) was produced, demonstrating anti-tumour and anti-metastatic activity in a diverse panel of P-cadherin–overexpressing tumour models, without introducing any adverse secondary effects in mice (Zhang *et al.*, 2010). This antibody failed to bind to the most closely target-related family members, including E-cadherin, N-cadherin, and VE-cadherin. PF-03732010 also reduced lymph node metastases and lowered the levels of circulating tumour cells (CTC) in whole blood of P-cadherin$^+$ tumour bearing mice. The anti-metastatic property of the antibody was remarkable, since it significantly inhibited tumour cell infiltration into the lungs. PF-03732010 still suppressed βctn, cyclin D1, Vimentin, Bcl-2, and survivin expression, decreased the Ki67 levels, and increased caspase-3 expression (Zhang *et al.*, 2010) (Fig. 4).

Taken together, these recent data highlight the critical role of P-cadherin signalling in regulating tumorigenesis and metastasis, especially because its inhibition leads to anti-tumour and anti-metastatic effects in target-associated tumour models without any adverse indication. These observations provide the rationale and guidance for the clinical development of PF-03732010, in which tumours with high P-cadherin expression will be essential criteria for patient selection. Future work is warranted to seek a reproducible method to quantify P-cadherin in human tumours and to find a reasonable cut-off of expression related with therapeutic response, in an attempt to reach the full potential for clinical development of the antibody. PF-03732010 is currently under Phase I clinical trial development.

Conclusions

Although this review is mainly focused on P-cadherin role as a poor prognostic factor, as well as a therapeutic target in breast cancer, its upregulation is also found in several other malignancies, affecting organs such as pancreas, stomach, bladder and prostate, where it is also associated with an aggressive phenotype and poor prognosis. Thus, antagonizing P-cadherin represents a novel approach for anticancer therapy, by targeting tumours with high P-cadherin expression. Interestingly, P-cadherin silencing induces significant growth inhibition in several tumour models tested; however, this anti-proliferative activity was never observed *in vitro* (Zhang *et al.*, 2010). This discrepancy suggests that fully functioning P-cadherin signalling may require the cell-cell and cell-stroma crosstalk in intact tumour architecture during tumorigenesis and metastasis, a process that may not be recapitulated under *in vitro* conditions and that should be further studied in the future.

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PUBLICATIONS

Paper 3

Oncogene (2009), 1–11

www.nature.com/onc

ORIGINAL ARTICLE

Extracellular cleavage and shedding of P-cadherin: a mechanism underlying the invasive behaviour of breast cancer cells

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Cell–cell adhesion is an elementary process in normal epithelial cellular architecture. Several studies have shown the role mediated by cadherins in this process, besides their role in the maintenance of cell polarity, differentiation and cell growth. However, during tumour progression, these molecules are frequently altered. In breast cancer, tumours that overexpress P-cadherin usually present a high histological grade, show decreased cell polarity and are associated with worse patient survival. However, little is known about how this protein dictates the very aggressive behaviour of these tumours. To achieve this goal, we set up two breast cancer cell models, where P-cadherin expression was differently modulated and analysed in terms of cell invasion, motility and migration. We show that P-cadherin overexpression, in breast cancer cells with wild-type E-cadherin, promotes cell invasion, motility and migration. Moreover, we found that the overexpression of P-cadherin induces the secretion of matrix metalloproteases, specifically MMP-1 and MMP-2, which then lead to P-cadherin ectodomain cleavage. Further, we showed that soluble P-cadherin fragment is able to induce *in vitro* invasion of breast cancer cells. Overall, our results contribute to elucidate the mechanism underlying the invasive behaviour of P-cadherin expressing breast tumours.

Oncogene advance online publication, 9 November 2009; doi:10.1038/onc.2009.338

Keywords: P-cadherin; migration; invasion; MMPs; sP-cad

Introduction

Cell migration and invasion are critical properties that characterize malignant neoplastic cells. Recently, a number of molecular mechanisms have been identified in transformed cells that become migratory and invasive during carcinogenesis. These includes alterations in cell–cell and cell–matrix adhesion, activation of small GTPases or modulation of receptor tyrosine kinase-mediated signal transduction pathways (Cavallaro and Christofori, 2004).

Cell–cell adhesion is crucial for the maintenance of normal epithelial cellular architecture and is frequently altered in tumour progression, inducing a multistep process termed epithelial-to-mesenchymal transition (EMT) (Thiery, 2002). During EMT, tumour cells progressively downregulate their cell–cell adhesion epithelial-specific proteins, such as E-cadherin, and express *de novo* mesenchymal adhesion molecules, such as N-cadherin. This cadherin switch leads to the inhibition of cell–cell contacts and elicits active signals, which support tumour cell migration, invasion and metastatic dissemination (Frixen *et al*., 1991; Chen *et al*., 1997). However, regardless the major progress in understanding the *in vitro* molecular processes underlying EMT and tumour progression, a number of questions remain unsolved. For instance, the majority of breast cancer subtypes are diagnosed as invasive and malignant under pathological criteria, but rarely loose complete E-cadherin expression and infrequently gain *de novo* N-cadherin expression (Sarrio *et al*., 2008).

Our group has been focused in studying the role of another classical cadherin in breast cancer, namely P-cadherin. As mentioned before, in breast cancer, many of the highly aggressive tumours do not actually show a cadherin switch. In contrast, these tumours overexpress P-cadherin, maintaining the normal E-cadherin expression (Paredes *et al*., 2005, 2008). In clinical terms, these P-cadherin-overexpressing tumours present high histological grade, with decreased cell polarity, aggressive behaviour and worse patient survival (Peralta Soler *et al*., 1999; Gamallo *et al*., 2001; Paredes *et al*., 2002, 2005). On the basis of these observations, we aimed to understand how P-cadherin, in an E-cadherin wild-type background, could influence the behaviour of these tumours. Thus, we have studied earlier the expression of classical cadherins in a small collection of breast cancer cell lines (Paredes *et al*., 2007), and we associated this expression with the invasiveness potential of these cells.

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Received 13 February 2009; revised 9 September 2009; accepted 16 September 2009

Indeed, it is possible to see that there is a significant association between P-cadherin overexpression and the invasion capacity of breast cancer cells that maintain the expression of wild-type E-cadherin.

In this study, we show for the first time that P-cadherin overexpression, in wild-type E-cadherin breast cancer cells, is able to induce increased cell invasion, motility and migration. Additionally, we found that the presence of P-cadherin is able to provoke the secretion of pro-invasive factors, such as matrix metalloproteases (MMPs), MMP-1 and MMP-2, which then lead to P-cadherin ectodomain cleavage. Interestingly, we observed that this formed soluble P-cadherin fragment (sP-cad) is responsible for the *in vitro* invasion of wild-type E- and P-cadherin expressing cells, which clarifies the mechanism associated to cell invasion and may explain the poor prognosis of patients harbouring breast tumours expressing these two molecules (Paredes *et al*., 2008).

Results

P-cadherin overexpression in wild-type E-cadherin breast cancer cell lines is associated with an increase in cell invasion

In a recent study, we analysed the mRNA expression levels of the major classical cadherins (E-, P- and N-cadherins) among a small collection of different breast cancer cell lines, using RT–PCR (Paredes *et al*., 2007). Comparing these results with the knowledge acquired about these cell lines in earlier publications (Figure 1a), especially concerning their invasiveness potential, EMT phenotype and gene expression profile (Charafe-Jauffret *et al*., 2006; Lombaerts *et al*., 2006; Neve *et al*., 2006; Blick *et al*., 2008), we can clearly see that E-cadherin mRNA is only expressed by cell lines that maintain an epithelial phenotype, whereas N-cadherin is expressed by cells that show a mesenchymal phenotype. Besides the phenotype, the expression of these cadherins is also highly correlated with cell invasiveness capacity, where E-cadherin-positive cell lines show low invasive potential, whereas N-cadherin expressing cells are widely described as highly invasive. When breast cancer cell lines are classified in accordance with their gene expression profile, E-cadherin is also predominantly expressed by both Luminal and Basal A cell lines, whereas N-cadherin transcripts are restricted to Basal B cell lines.

Although these associations are already well known for E- and N-cadherin, similar findings were never found, concerning P-cadherin expression and these cell properties. Looking carefully to these associations (Figure 1a), P-cadherin mRNA transcripts are predominantly detected in E-cadherin-positive cells, being mostly associated with an epithelial phenotype. Curiously, this cadherin is strongly expressed by cell lines classified as Basal A, being also associated with an increased cell invasive capacity, compared with other epithelial cell lines harbouring a Luminal gene expression profile. Using Matrigel invasion assay, we confirmed the invasion potential of two Luminal cell lines, namely MCF-7/AZ and T47D, and two Basal A cell lines, MDA-MB-468 and BT-20. Figure 1b shows that cell lines with higher levels of P-cadherin expression revealed an increased ability to invade through Matrigel. More importantly, these results are in agreement with what has been described in invasive primary breast tumours, showing the clinical relevance of P-cadherin expression in the diagnosis and prognosis of patients with aggressive mammary carcinomas (Peralta Soler *et al*., 1999; Paredes *et al*., 2005).

To determine whether or not P-cadherin expression is in part responsible by the moderate/high invasive capacity of Basal A cells lines, we set up two E-cadherin-positive cancer cell models: (1) the stable induction of P-cadherin overexpression in non-invasive MCF-7/AZ cells, by retroviral infection; and (2) the transient silencing of P-cadherin in invasive BT-20 cells, using specific small interference RNA (siRNA). As can be noticed in Figure 2a, these two cell models were established and the role of P-cadherin in cell invasion was, therefore, analysed. P-cadherin-overexpressing MCF-7/AZ cells showed a significant increase in cell invasion capacity, which was repressed when cells were treated with a P-cadherin inhibitory-function antibody, pointing to a P-cadherin-dependent cell invasion mechanism (Figure 2b). Identically, BT-20 invasive potential was significantly inhibited when P-cadherin was silenced by siRNA (Figure 2c).

As a conclusion, we can state that P-cadherin-overexpressing breast cancer cells, although maintaining its epithelial phenotype, have selective advantage to invade when compared with cell lines that mostly express E-cadherin.

P-cadherin overexpression promotes cell motility and cell migration of breast cancer cells

To further explore the function of P-cadherin in breast cancer, its effect on cell motility and cell migration was also tested.

We evaluated breast cancer single cell motility by time-lapse microscopy, during 6 h of culture (see movies in Supplementary data). Figure 3 represents snapshot images from time-lapse movies, where differences were seen between the motility of MCF-7/AZ.Mock and MCF-7/AZ.Pcad cells. Figure 3 also shows the results obtained with BT-20 cells transfected with a control siRNA or with an siRNA that specifically abolish P-cadherin expression. The trajectory of the cell's nuclei from both cell lines is showed in Figure 3a. MCF-7/AZ.Mock cells barely showed significant movement, whereas MCF-7/AZ.Pcad cells exhibited increased cell motility. Besides this significant increase in the total number of cells with motile capability, MCF-7/AZ.Pcad cells also showed a fourfold increase in cell speed (Figure 3b). P-cadherin silencing in BT-20 cells, turns these cells significantly less motile, and the cell speed was decreased for half of the one showed by BT-20 control cells (Figures 3c and d).

a

Cell line	E-CAD	P-CAD	N-CAD	Invasiveness (1,2)	EMT phenotype (3-5)	Gene expression profile (6)
SKBR3	-	-	-	Low	Epithelial	Luminal
ZR-75.1	++	+	-	Low	Epithelial	Luminal
MCF-7/AZ	++	+	-	Low	Epithelial	Luminal
T47D	++	+	-	Low	Epithelial	Luminal
BT-474	++	+	-	Low	Epithelial	Luminal
MDA-MB-468	++	++	-	Mod	Epithelial	BasalA
BT-20	++	+++	-	Mod/High	Epithelial	BasalA
BT-549	-	+	++	High	Mesenchymal	Basal B
HS578T	-	+	+++	High	Mesenchymal	Basal B
MDA-MB-231	-	-	+	High	Mesenchymal	Basal B
MDA-MB-435	-	-	+	High	Mesenchymal	Basal B
HBL-100	-	-	++	High	Mesenchymal	Basal B

1) Paredes et al, 2007; 2) Blick et al, 2008; 3) Perou et al, 2000; 4) Lombaerts et al, 2006; 5) Charafe-jauffret et al, 2006; 6) Neve et al, 2006

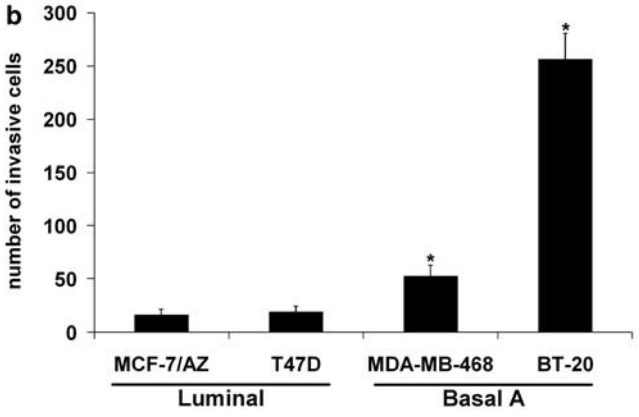

Figure 1 Association between P-cadherin expression and cell invasiveness in wild-type E-cadherin breast cancer cell lines. (**a**) Table comparing the pattern of expression of classical cadherins (namely E-, P- and N-cadherin) in different breast cancer cell lines with their invasiveness potential, EMT (epithelial-to-mesenchymal transition) phenotype and gene expression profile. (**b**) Matrigel invasion assay was used to analyse the invasion potential of two Luminal cell lines, MCF-7/AZ and T47D and two Basal A cell lines, MDA-MB-468 and BT-20. The basal cell lines showed significant increase in cell invasion, when compared with the Luminal cell lines (*$P<0.005$ compared with MCF-7/AZ non-invasive cells).

Besides analysing cell motility, we evaluated cell migration capacity, using a wound-healing migration assay. As observed in Figures 4a and b, P-cadherin-positive cells migrated significantly faster into the wound compared with control cells; importantly, this result was not due to an increase in cell proliferation rate of P-cadherin-overexpressing cells (Figure 4c). To confirm that induced migration was dependent of P-cadherin expression in these cells, the same experiment was performed in the presence of a functionally blocking antibody against P-cadherin activity (the NCC-CAD-299 clone). As shown in Figure 4b, the presence of this antibody significantly inhibited the migration capacity of P-cadherin-overexpressing cells into the wound area, whereas a non-specific IgG did not. An obvious observation of P-cadherin-induced cell migration was the evident pattern of collective cell migration, as P-cadherin-overexpressing cells do not lose their E-cadherin cell–cell contacts, and cells migrate faster and cohesive. Time-lapse microscopy movies clearly show this result (see Supplementary data).

In both assays, besides the increase in cell motility and migration, it was also possible to observe clear differences between cell's phenotype (see Supplementary data), where P-cadherin-overexpressing cells presented an increased number of membrane protrusions, structures that are usually associated to moving cells.

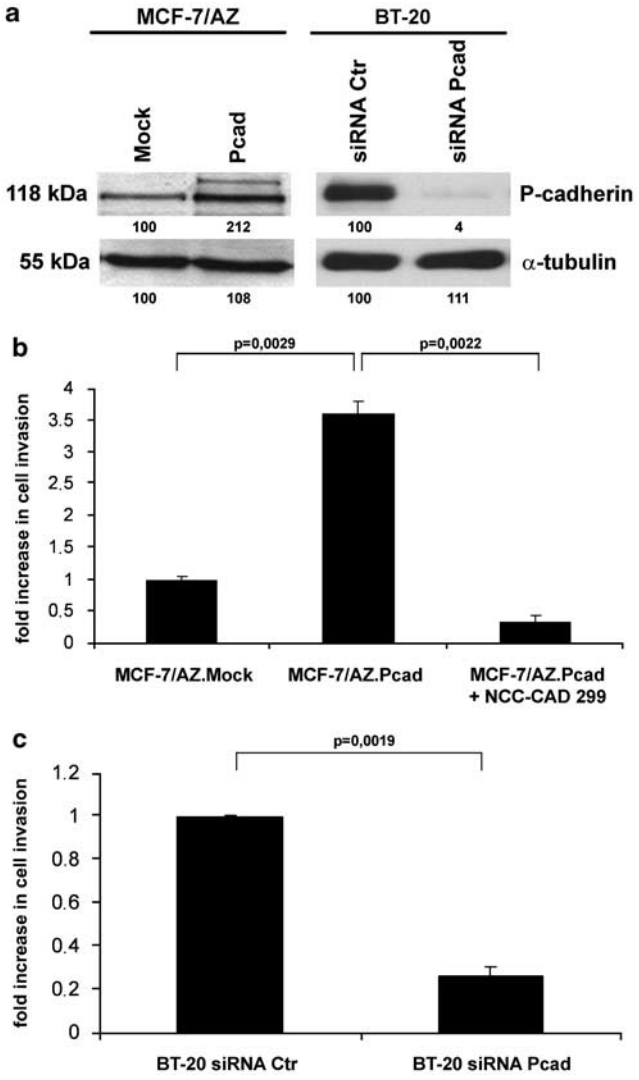

Figure 2 P-cadherin is involved in breast cancer cell invasion. (**a**) P-cadherin and α-tubulin protein expression in two different breast cancer cell models: induction of P-cadherin overexpression in MCF-7/AZ cells, by retroviral infection, and silencing of P-cadherin overexpression in BT-20 cells, using specific small interference RNA (siRNA). (**b**) Matrigel invasion assay of control MCF-7/AZ.Mock cells, and MCF-7/AZ.Pcad cells in the absence or presence of a function-blocking anti-P-cadherin antibody (NCC-CAD-299, $P=0.0029$ and 0.0022, respectively). (**c**) Matrigel invasion assay of BT-20 siRNA control cells compared with BT-20 with P-cadherin knockdown expression ($P=0.0019$).

P-cadherin overexpression induces the formation of cell membrane protrusive structures

An effective cell migration requires the integration of localized and transient signalling events, leading to changes in cellular architecture, namely in the re-organization of the actin cytoskeleton. Given that P-cadherin affects cell migration, motility and invasion, we analysed the effect of P-cadherin overexpression on the actin cytoskeleton organization, by F-actin fluorescence immunostaining. Differences between control and MCF-7/AZ.Pcad cells were evident as P-cadherin-overexpressing cells showed an increase in membrane ruffling and in actin cellular extensions, as well as in their cytoplasmic area (Figure 5). Indeed, P-cadherin-overexpressing cells, including MCF-7/AZ.Pcad and BT-20, show an upper cell localized nuclei and appear as rounded and flat cells with actin-rich sheet-like membrane protrusive structures that, according with the literature, are essentially observed during crawling cell motility and spreading (Yamazaki *et al*., 2005; Chhabra and Higgs, 2007).

Using double F-actin and p120ctn staining, MCF-7/AZ.Mock versus MCF-7/AZ.Pcad cells showed distinct aggregation ability, where MCF-7/AZ.Mock cells, in contrast to P-cadherin-overexpressing cells, showed well-formed and tight aggregates (Figure 5). Indeed, microscopic fluorescence imaging of P-cadherin expressing cells showed that P-cadherin destabilizes cell–cell adhesion, promoting cytoskeleton changes, leading to a different cell phenotype. The cells resemble the aggressive morphology observed in primary basal-like P-cadherin-positive breast carcinomas, as these cells normally acquire a large cytoplasm and several membrane protrusions, and cell–cell adhesion is not usually mediated by a compact zipper-like structure (Yamazaki *et al*., 2005; Chhabra and Higgs, 2007).

These results clearly show an association between P-cadherin expression and actin cytoskeleton reorganization, suggesting that P-cadherin has a role in the mechanism that regulates the cellular architecture changes that are needed to promote cell migration and invasion.

P-cadherin overexpression induces active MMPs that are responsible for its extracellular cleavage and shedding

One of the families of proteases, which are well known to be involved in cell invasion induction, namely in the extracellular matrix (ECM) degradation process, is the family of MMPs. On the basis of this, MMP activity levels were assessed in the conditioned medium from P-cadherin-overexpressing breast cancer cells, using β-casein and gelatin zymography. The results obtained showed that P-cadherin expression induces significantly the levels of active MMP-1 (42 kDa) and active MMP-2 (66 kDa) in the conditioned medium compared with control cells (Figure 6a). These observations were further confirmed by western blot, using antibodies specifically to analyse the expression of inactive and active forms of MMP2 and MMP1 (Figure 6b). Accordingly, also high levels of active MMP-1 and MMP-2 were found in the conditioned medium from BT-20 cells, although transient P-cadherin silencing was not enough to abrogate the expression levels of these active MMP forms (data not shown).

Given that MMPs were already described to shed the extracellular domains of membrane glycoproteins, including E-cadherin, giving rise to a soluble fragment with pro-invasive activity (Lochter *et al*., 1997; Herren *et al*., 1998; Noe *et al*., 2001), we looked forward for the presence of soluble fragments of cadherins in the conditioned medium from the studied cell lines. Surprisingly, no significant differences were observed

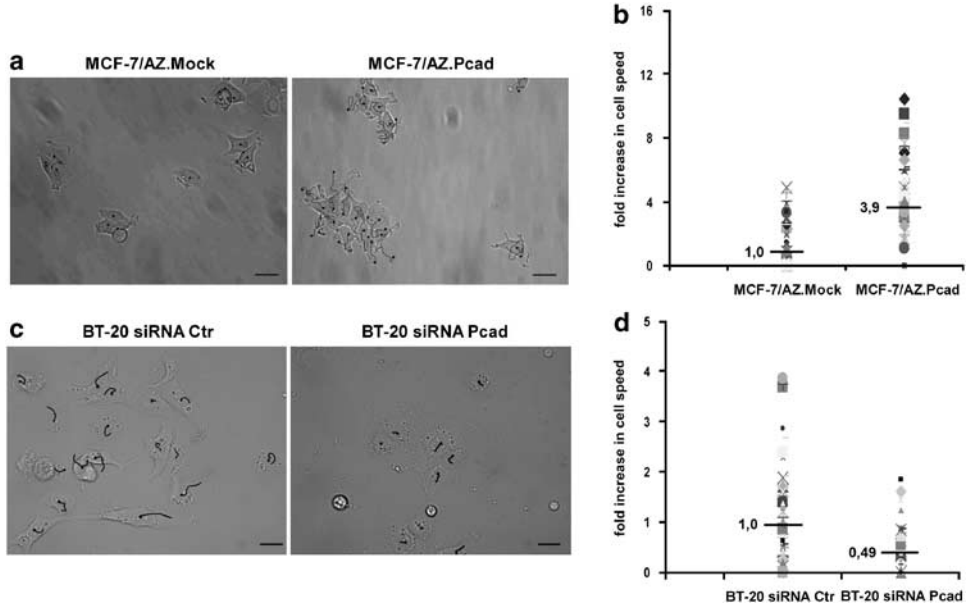

Figure 3 Promotion of cell motility by P-cadherin overexpression in breast cancer cells. Snapshot images from time-lapse movies of MCF-7/AZ.Mock and MCF-7/AZ.Pcad cells (**a**) and BT-20 siRNActr and BT-20 siRNAPcad (**c**). Cells were monitored during 6 h, and trajectories for each cell were determined manually based on the centre of the nuclei along time. Black lines represent the trajectories performed by cells. Cell speed, considering the ratio from the distance made by cells versus the time of movement, was quantified for MCF-7/AZ.Mock and MCF-7/AZ.Pcad cells (**b**) and for BT-20 siRNActr and BT-20 siRNAPcad (**d**), where 300 cells were analysed per cell line ($P<0.001$). In both cell models, is possible to conclude that P-cadherin expression promotes an increase in cell motility and cell speed.

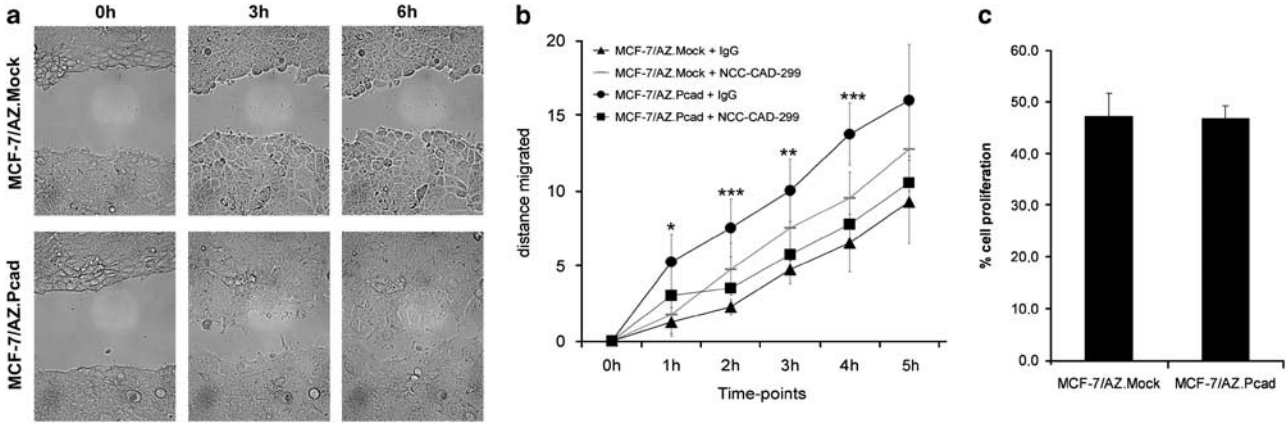

Figure 4 Induction of cell migration by P-cadherin overexpression in breast cancer cells. (**a**) Cell migration was estimated by means of wound-healing migration assay, and monitored by time-lapse microscopy. The distances migrated by breast cancer cells were measured at several time points: 0, 1, 2, 3, 4, 5 and 6 h. The experiment shown is relative to a representative experiment that was repeated three times. (**b**) Migration of MCF-7/AZ.Mock and MCF-7/AZ.Pcad cell lines, in the presence or absence of a function-blocking anti-P-cadherin antibody (NCC-CAD-299) was evaluated by wound-healing migration assay (*$P<0.05$, corresponding to P-values from MCF-7/AZ.Pcad + IgG compared with MCF-7/AZ.Mock + IgG cells; **$P<0.05$, ***$P<0.01$, corresponding to P-values from MCF-7/AZ.Pcad + IgG compared with MCF-7/AZ.Pcad + NCC-CAD-299 antibody treatment). P-cadherin expression is directly implicated in the migratory capabilities of this cell line. (**c**) BrdU proliferation assay was performed in MCF-7/AZ.Mock and MCF-7/AZ.Pcad cells, to exclude that differences observed in cell migration were due to altered cell proliferation. No differences were observed in the percentage of cell proliferation in both cell lines.

concerning the presence of soluble E-cadherin (sE-cad); however, sP-cad was increased 8.7-fold in the conditioned medium from MCF-7/AZ.Pcad cells (Figure 6c). BT-20 cells also showed high levels of sP-cad, which were significantly decreased when these cells were transfected with P-cadherin siRNA (Figure 7a). These results showed, as expected, a direct association between full-length P-cadherin overexpression at the cell membrane and the presence of sP-cad in the conditioned media.

Finally, our goal was to assess if MMPs might have a role in P-cadherin shedding, as was described earlier for

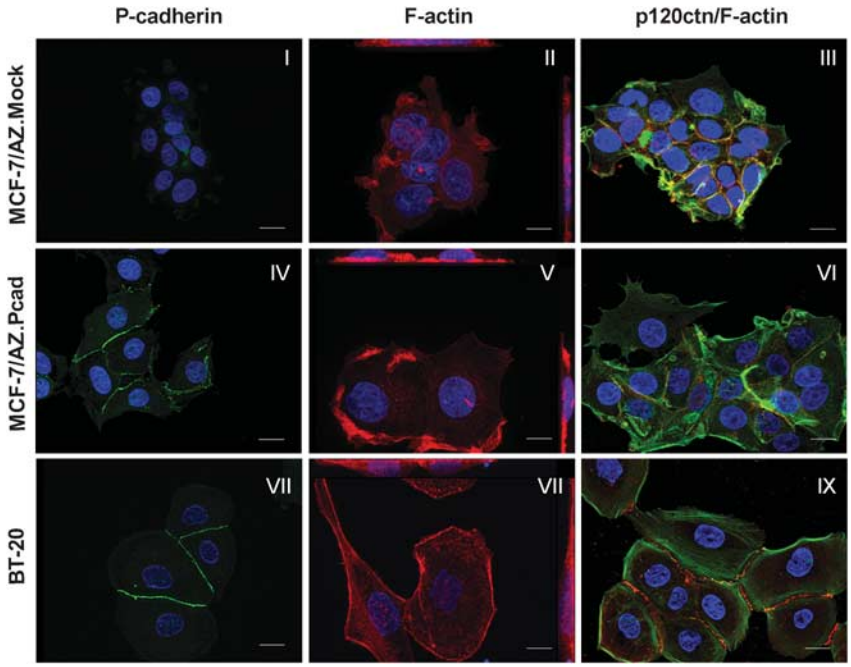

Figure 5 P-cadherin overexpression induces the formation of cell membrane protrusions and large cytoplasm. Breast cancer cells were seeded on a glass surface, fixed and stained for P-cadherin (I, IV, VII). FITC-phalloidin staining was also performed (II, V, VII), to visualize actin filaments (1000 × amplification), where it was possible to confirm alterations in the actin cytoskeleton re-organization in P-cadherin-overexpressing cells. Confocal microscopy imaging of aggregates from MCF-7/AZ.Mock (III), MCF-7/AZ.Pcad (VI) and BT-20 cells (IX), stained for F-actin (green) and p120ctn (red) (400 × amplification) was performed. Cell phenotype and structure of cell clusters were different, attributing a role for P-cadherin in the induction of loose aggregates. The white line in each picture represents 20 μm scale.

E-cadherin. Thus, MCF-7/AZ.Pcad cells were treated with a MMP inhibitor (with higher affinity to inhibit MMP-1 and MMP-2), the conditioned medium was recovered and the presence of sP-cad was evaluated by western blot. Remarkably, the treatment with the MMP inhibitor reduced the levels of sP-cad, showing that MMPs have an important role in P-cadherin cleavage and shedding (Figure 6d). To understand if P-cadherin shedding had also a role in the production of MMPs, inducing a mechanism of positive feedback, the parental cell line MCF-7/AZ was treated with human recombinant P-cadherin (hrP-cad). This peptide should mimic the effect of sP-cad, as it only harbours the extracellular part of this complete adhesion molecule. Interestingly, we found that hrP-cad induced the secretion of active MMP-1 and MMP-2, as happens in MCF-7/AZ.Pcad cells (Figure 6e). We also showed that hrP-cad is also cleaved by MMPs, originating an 80 kDa fragment, identical to sP-cad; the levels of this fragment were decreased when cells were treated with hrP-cad in combination with MMP inhibitor, confirming the importance of these MMPs to full-length P-cadherin cleavage (Figure 6f).

These results show that P-cadherin overexpression, sP-cad cleavage, as well as active MMPs secretion, are highly correlated. Briefly, P-cadherin expression leads to the secretion of active MMPs, enzymes that then cleave the extracellular domain of P-cadherin, giving rise to sP-cad. This soluble fragment is able to induce and maintain the secretion of active MMPs.

sP-cad has pro-invasive activity in breast cancer cells

As a crucial step for invasion and metastasis is the destruction of biological barriers (basement membrane and ECM) by activated proteolytic enzymes, we aimed to assess whether the medium from P-cadherin-overexpressing breast cancer cells, rich in MMPs and sP-cad, was enough to facilitate cell invasion of non-invasive cells. Thus, we performed the same invasion assay using the parental MCF-7/AZ cell line, treated with the conditioned medium obtained from MCF-7/AZ.Mock and MCF-7/AZ.Pcad cells (Figures 7a and b). Interestingly, only the conditioned medium from P-cadherin-overexpressing cells itself was able to significantly induce invasion. This effect was not observed in the presence of the conditioned medium secreted by cells without P-cadherin overexpression (MCF-7/AZ.Mock). To confirm these results, these same cells were treated with the conditioned medium from BT-20 cells, which also contain high levels of sP-cad and MMPs, and '*de novo*' invasive cell behaviour was observed. These results show that P-cadherin-overexpressing cells secrete factors to the medium that are crucial for cell invasion induction (Figures 7a and b).

In the past, *in vitro* studies showed that sE-cad has a pro-invasive role in tumour cell lines, although its mechanism of action is still not well understood (Noe *et al*., 2001). However, the potential activity of sP-cad in cell invasion induction has never been described in *in vitro* studies. To understand whether sP-cad would have a role in breast cancer cell invasion, we depleted the

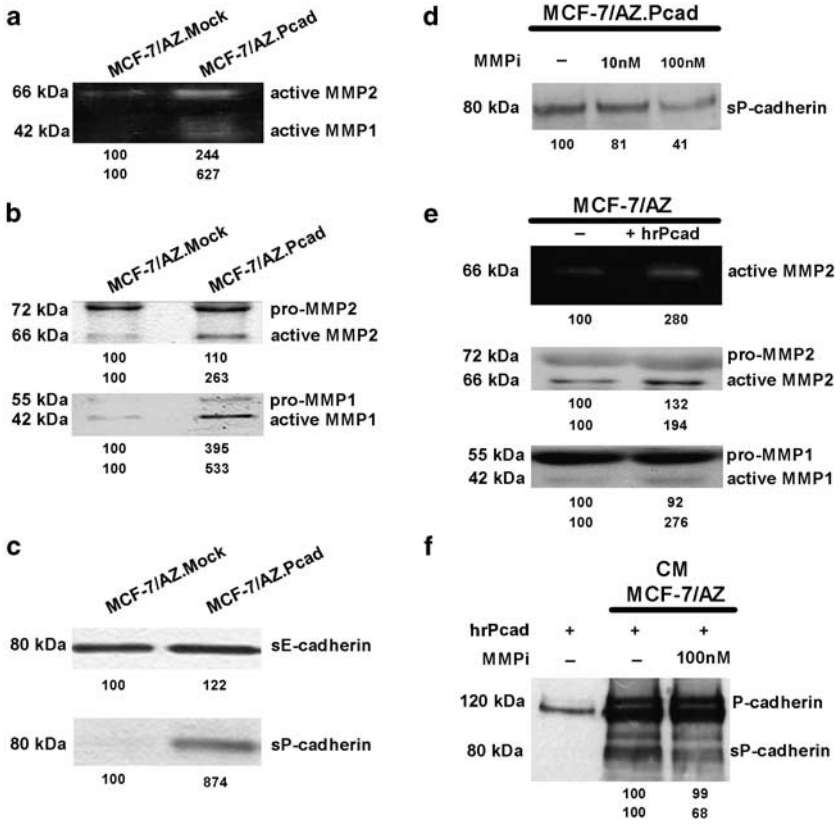

Figure 6 P-cadherin overexpression induces increased levels of MMP-1 and MMP-2 activity, which have a role in P-cadherin shedding. (**a**) β-casein zymography, which allows identification of activity of MMP-1 and MMP-2, was performed using the conditioned medium from mock and MCF-7/AZ.Pcad cells, cultured in collagen type-I. (**b**) Western blot to detect the secretion of active-MMP-1 and active-MMP-2 into the conditioned medium was evaluated and compared between mock and P-cadherin-overexpressing cells. (**c**) Detection for the presence of sE-cadherin and sP-cadherin fragments in the medium was achieved by performing western Blot for these proteins, using the conditioned medium obtained from each cell line. (**d**) Effect of MMPs in the shedding of P-cadherin was evaluated by analysing the presence of the sP-cad fragment in the conditioned medium from MCF-7/AZ.Pcad cells treated with different concentrations of the MMP inhibitor III. (**e**) Zymography detecting increased MMP-2 activity levels in parental MCF-7/AZ cells treated with a human recombinant fragment of P-cadherin (hrP-cad). Western blot for active-MMP-1 and active-MMP-2 present in the conditioned medium from control cells treated with hrP-cad. (**f**) Protein expression analysis for the conditioned medium from control cells treated with hrP-cad and MMPi, to confirm that hrP-cad can be shed to an 80 kDa form and that this process is MMP dependent.

sP-cad fragment from the conditioned medium of MCF-7/AZ.Pcad cells by repeated immunoprecipitations, using a specific P-cadherin antibody (Figures 7a and b). Moreover, we treated the cells with hrP-cad, which was added to the conditioned medium collected from MCF-7/AZ.Mock cells. Afterwards, we performed Matrigel invasion assay using the parental non-invasive MCF-7/AZ cells, treated with these mediums: or with the hrP-cad-rich medium or with the depleted (without sP-cad) conditioned medium of P-cadherin-overexpressing cells. Although hrP-cad-rich medium promoted cell invasion capacity to a non-invasive cell line, this effect was completely abolished when sP-cad was depleted from the medium (Figures 7a and b). Moreover, when MCF-7/AZ cells were treated with the conditioned medium from BT-20 cells, where P-cadherin was silenced by siRNA, its invasion was significantly reduced comparing with BT-20 control cells. Together, these results clearly show a critical role for sP-cad in breast cancer cell invasion.

Finally, to understand whether the invasion effect induced by sP-cad was due to homotypic interactions with endogenous P-cadherin on the surface of the cells, we decided to measure the invasion rates of BT-20 target cells, with P-cadherin siRNA knockdown, exposed to conditioned medium rich in sP-cad or rich in hrP-cad (Figure 7c). Interestingly, we found that in both situations, it is possible to restore the invasion capacity of these cells. These results show that the invasive effect mediated by sP-cad is not due to a homotypic interaction with endogenous P-cadherin at the cell membrane.

Discussion

Previous studies have shown that P-cadherin expression in breast carcinomas is able to identify a subgroup of lesions with a more aggressive behaviour and poor

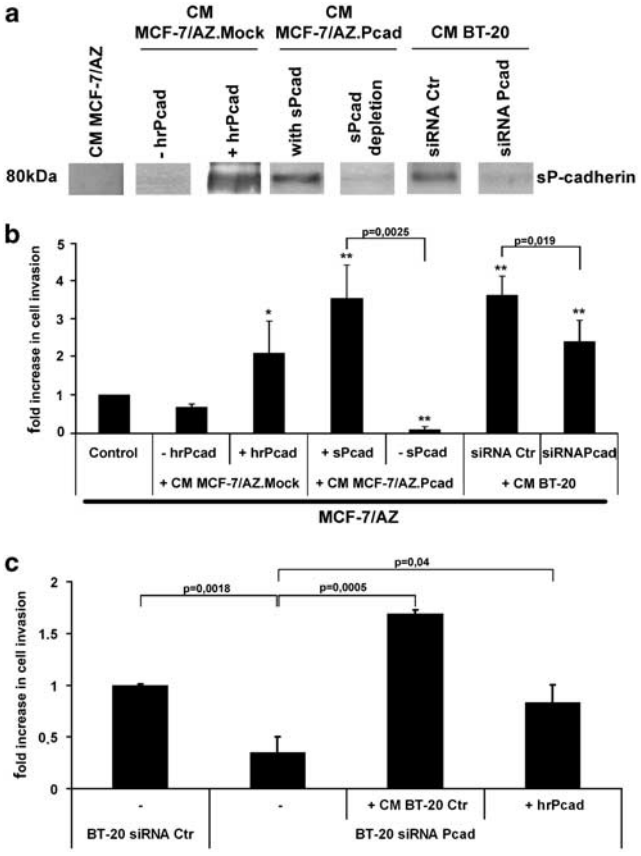

Figure 7 sP-cad has pro-invasive activity. (**a**) Western blot for several conditioned medium, showing different levels of sP-cad. Conditioned medium from MCF-7/AZ.Pcad cells (with sP-cad) was subjected to serial immunoprecipitations for the sP-cad fragment, using a specific monoclonal P-cadherin antibody, originating the conditioned medium with sP-cad depletion. (**b**) To clarify the specific role of s-Pcad in breast cancer cell invasion, matrigel invasion assay was performed in non-invasive parental MCF-7/AZ cells incubated with the conditioned medium from the different cell lines that present different levels of sP-cad (shown in **a**). Control cells were treated conditioned medium from MCF-7/AZ.Mock cells (with and without hrP-cad), conditioned medium from MCF-7/AZ.Pcad cells (with and without sP-cad depletion) and with conditioned medium from BT-20 cells (with and without P-cadherin knockdown). Student's *t*-test was performed and the statistically different values are indicated ($*P<0.05$ and $**P<0.01$ compared to control conditioned medium). (**c**) To understand whether the invasion effect induced by sP-cad was due to homotypic interactions with endogenous P-cadherin on the surface of the cells, we calculated the invasion rates of BT-20 with P-cadherin knockdown, exposed to conditioned medium from BT-20 control cells (rich in sP-cad) or rich in hrP-cad. We observed a rescue in the invasion capacity of these cells.

patient survival (Peralta Soler *et al*., 1999; Gamallo *et al*., 2001; Paredes *et al*., 2002, 2004, 2005, 2007, 2008). Similar associations between P-cadherin expression and poor prognosis are described in other cancer models, namely endometrial, cervical, gastric and pancreatic carcinomas (Stefansson *et al*., 2004; Longatto Filho *et al*., 2005; Taniuchi *et al*., 2005).

Our group has previously shown *in vitro* that, using HEK 293 T cells as a model system, P-cadherin has a pro-invasive activity, through its juxtamembrane domain (Paredes *et al*., 2004). In contrast, in highly invasive melanoma cell lines, P-cadherin overexpression is able to promote the formation of cell–cell contacts and counteract invasion (Van Marck *et al*., 2005), showing that it may function either as an invasion promoter or as an invasion suppressor depending on tissue specificity. On the basis of clinical evidences and on its contradictory biological role in distinct tumour tissues, we aimed at understanding how P-cadherin influences tumour aggressiveness. Furthermore, we aimed to clarify, besides cell invasion, its role in migration, motility and activation of MMPs, using breast cancer-derived cell lines.

Using *in vitro* cell models, we found for the first time that overexpression of exogenous P-cadherin is able to promote single cell motility, inducing an increase in the number of moving cells and speed when compared with cells with low levels of this protein. Furthermore, P-cadherin-overexpressing cells not only showed increased single cell motility, but also increased directional cell migration, as well as, invasion capacity through the Matrigel. This behaviour was shown to be directly dependent on P-cadherin, as when overexpressing cells were treated with a P-cadherin blocking antibody or transfected with a siRNA to inhibit P-cadherin transcripts, there was an inhibition of both effects (migration and invasion). Interestingly, Simpson *et al*. (2008) also identified CDH3 (the P-cadherin codifying gene) as one of the genes involved in the regulation of breast cell migration using an siRNA approach. Further, in other studies, using different cancer cell models, P-cadherin was shown to have a role in promoting cell migration. Namely, Taniuchi *et al*. (2005) showed that a pancreatic cancer cell line, transfected with wild-type P-cadherin, migrated faster than the cells without this molecule. Epithelial cell migration requires the coordination of three basic cellular processes: actin cytoskeleton reorganization, matrix adhesion and matrix re-modelling (Lauffenburger and Horwitz, 1996; Fenteany *et al*., 2000). In this present study, we also show, by time-lapse microscopy and actin phalloidin staining, that P-cadherin is able to induce phenotypic changes involving alterations in cell polarity and leading edge morphology, formation of membrane protrusions, as well as, increase of their cytoplasmic area, which usually is characteristic from cells with a motile behaviour.

Further, we aimed to determine the molecular mechanisms underlying P-cadherin overexpression and its cellular-associated effects in breast cancer cells. It has been described that the degradation of the ECM, with recruitment of proteolytic enzymes, occurs in a variety of cellular events requiring tissue reorganization, such as embryonic development, wound healing and cancer progression (Vu and Werb, 2000). Among these enzymes, MMPs are able to degrade almost all the ECM components, and have largely been involved in both tumour invasion *in vitro* and in early and late stages of tumour progression *in vivo*. When we evaluated the cell invasion capacity of non-invasive parental cells exposed to conditioned media from P-cadherin-over-

expressing cells, we conclude that indeed P-cadherin induces the secretion of factors that facilitate cell invasion of non-invasive MCF-7/AZ cells that we identified as being active forms of MMP-1 and MMP-2. More importantly, we showed that these enzymes are responsible by the shedding of a soluble extracellular fragment of P-cadherin harbouring pro-invasive activity, as its depletion from the conditioned medium of P-cadherin-overexpressing cells re-establish the non-invasive phenotype of E-cadherin breast cancer cells.

Our results are in accordance with several recent studies, concerning the role of MMP-2, MMP-1 and sP-cad in breast cancer. *In vivo*, MMP-2 is described as a key enzyme for the degradation of the ECM and facilitating tumour invasion and metastasis, being its active form present in half of all human breast carcinomas (Stetler-Stevenson *et al*., 1993; Remacle *et al*., 1998). Not only MMP-2 has pro-invasive activities, but also MMP-1 overexpression has been shown in a variety of advanced carcinomas, being associated with poor prognosis (Murray *et al*., 1998a, b; Ito *et al*., 1999; Fujimoto *et al*., 2008; Okuyama *et al*., 2008). High levels of MMP-1 expression have been detected in human breast cancer cells with elevated metastatic capacity towards the bone, providing evidence for its role in cancer cell invasion and metastasis (Kang *et al*., 2003; Okuyama *et al*., 2008). However, the molecular mechanism by which P-cadherin expression induces the secretion of these both enzymes to the medium remains unsolved.

The mechanism of ectodomain cleavage of adhesion proteins, mediated by MMPs, has been already well described (Lochter *et al*., 1997; Herren *et al*., 1998; Noe *et al*., 2001). MMP-3 and MMP-7 have a role in the shedding of the extracellular domain of E-cadherin, generating a soluble 80 kDa fragment (Noe *et al*., 2001). Interestingly, the sE-cad fragment, when released, inhibits E-cadherin functions in a paracrine way, inducing cell invasion into collagen type I and inhibiting E-cadherin-dependent cell–cell aggregation (Noe *et al*., 2001). Very recently, Mannello *et al*. (2008) showed a significant increased shedding of soluble fragments of P-cadherin in nipple aspirate fluids from women with breast cancer, when compared with healthy subjects or with women with pre-cancer conditions. This suggests its possible release through proteolytic processing in cancer cells. Until nowadays, there are only few studies showing the presence of sP-cad in biological human fluids (such as milk (Soler *et al*., 2002) and sperm (De Paul *et al*., 2005)), but no specific cellular effect has been attributed to this fragment. Our results show, for the first time, that sP-cad is produced by proteolytic enzymes (MMP-1 and MMP-2) and is responsible for the invasive capacity of breast cancer cells. Moreover, this invasive effect induced by sP-cad is independent from the endogenous expression of P-cadherin at the cell membrane. Further research is needed to elucidate which signalling pathways are activated by this pro-invasive factor.

In conclusion, the results herein described contribute to clarify the role of P-cadherin expression in breast cancer, as they unravel the molecular mechanism and the associated cellular effects mediated by this protein. In breast cancer cells, P-cadherin expression gives an advantage to cells to migrate and move, as well as the possibility to secrete pro-invasive factors, such as MMPs and sP-cad.

Materials and methods

Cell culture and transfection

Human cancer cell lines were obtained as described: MCF-7/AZ (kindly given by Prof. Marc Mareel, Ghent University, Belgium), T47D, MDA-MB-468 and BT-20 from American Type Culture Collection (Manassas, VA, USA). Cell lines were routinely maintained at 37 °C, 5% CO_2, in the following media (Invitrogen Ltd, Paisley, UK): 50% DMEM/50% HamF12 (MCF-7/AZ), DMEM (BT-20, T47D, MDA-MB-468). All the media contained 10% heat-inactivated fetal bovine serum (Greiner bio-one, Wemmel, Belgium), 100 IU/ml penicillin and 100 μg/ml streptomycin. MCF-7/AZ cell line was retrovirally stable transduced to encode P-cadherin (MCF-7/AZ.Pcad cell line), as described earlier (Paredes *et al*., 2004). MCF-7/AZ.Mock cell line, encoding only EGFP, was used as a control. BT-20 transient transfection with siRNA specific for P-cadherin (50 nM, Hs_CDH3_6, GW Validated siRNA, Qiagen, Cambridge, MA, USA) was carried out using Lipofectamine 2000 (Invitrogen), according to the manufacturer's procedures. A negative control, with no homology to any gene, was also used (Qiagen).

For the conditioned medium assays, cells were grown until confluence in collagen-type I-coated flasks (0.2 mg/ml—Sigma, Steinheim, Germany) and incubated in serum-free medium for 48 h. The conditioned medium was filtered and the proteins secreted were quantified in the recovered supernatant.

Antibodies and chemicals

Primary antibodies. P-cadherin (Western blot: clone 56, BD Biosciences, Lexington, KY, USA; immunofluorescence: Cell Signalling tecnhology, Boston, MA, USA; P-Cadherin-blocking-function antibody: clone NCC-CAD-299, Zymed Laboratories, San Francisco, CA, USA), E-cadherin (clone HECD-1, Takara Bio Inc., Shiga, Japan), p120ctn (clone 98, BD Biosciences), β-actin (I-19, Santa Cruz Biotechnologies, CA, USA), MMP-1 and MMP-2 (Ab-6 and Ab-7 respectively, Neomarkers, Fremont, CA, USA).

Chemicals. MMP inhibitor III (treatement with 10 nM or 100 nM, for 48 h, Calbiochem, EMD Chemicals, Darmstadt, Germany); Recombinant human P-cadherin/Fc chimaera (20 μg/ml; hrPcad, R&D Systems, Inc., Minneapolis, MN, USA). For detailed description see Supplementary data.

Cancer cell motility and wound-healing assay

For the motility assay, cells were monitored with an inverted time-lapse controller (Leica FW 4000, DMIRE 2, Pecon, Leica, Bensheim, Germany) and distance moved by the cells was determined, as well as cell speed (μm/h).

For the wound-healing migration assay, wounds were made across the cell monolayer and distances between the wound edges were determined. Cells were also treated with NCC-CAD-299 (100 μg/ml) or control mouse immunoglobulin G (IgG; 100 μg/ml, Upstate, Millipore, Billerica, MA, USA).

BrdU proliferation assay was performed to exclude that difference in cell migration was due to cell proliferation. For detailed description see Supplementary data.

Immunofluorescence and confocal microscopic analysis
Cells were plated on glass coverslips (Becton Dickinson Labware, Franklin Lakes, NJ, USA), fixed with 4% formaldehyde. For protocol details see Supplementary data.

Gelatin and β-casein zymography
The conditioned medium was analysed for proteinases activity using gelatin and β-casein zymography (gels loaded with 12 and 100 μg of protein, respectively) as described earlier (Oliveira *et al*., 2003). Quantification of band density was done using the Quantity One software (version 4.0, Bio-Rad, Hercules, CA, USA), unless specified.

Immunodepletion and western blot
For sP-cad immunodepletion, 300 μl of conditioned medium collected from MCF-7/AZ.Pcad cells was incubated with NCC-CAD-299 and immunoprecipitated twice. Western Blot was performed as described earlier (Paredes *et al*., 2007). The experiments selected to show are representative ones. For more detailed description on immunoprecipitation and western Blot see Supplementary data.

Matrigel invasion assay
Matrigel invasion assay was performed according to manufacter's instrutions (BD Biosciences). For more detailed description see Supplementary data.

Statistical analysis
Data are expressed as mean values of at least three independent experiments ± s.d. Student's *t*-tests were used to determine statistically significant differences ($P<0.05$).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The work presented was mainly supported by a scientific project (POCI/BIA-BCM/59252/2004) financed by the Portuguese Science and Technology Foundation (FCT). FCT also provided research grants as follows: Programa Ciência 2007 (FCT) for Joana Paredes, and PhD research grants for Ana Sofia Ribeiro (SFRH/BD/36096/2007) and André Albergaria (SFRH/BD/15316/2005).

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

PUBLICATIONS

Paper 4

HMG Advance Access published April 21, 2010

Human Molecular Genetics, 2010 **1–13**
doi:10.1093/hmg/ddq134

ICI 182,780 induces P-cadherin overexpression in breast cancer cells through chromatin remodelling at the promoter level: a role for C/EBPβ in *CDH3* gene activation

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Received February 3, 2010; Revised and Accepted April 5, 2010

***CDH3*/P-cadherin is a classical cadherin. Overexpression of which has been associated with proliferative lesions of high histological grade, decreased cell polarity and poor survival of patients with breast cancer. *In vitro* studies showed that it can be up-regulated by ICI 182,780, suggesting that the lack of ERα signalling is responsible for the aberrant P-cadherin overexpression and for its role in inducing breast cancer cell invasion and migration. However, the mechanism by which ER-signalling inhibition leads to P-cadherin expression is still unknown. The aim of this study was to explore the molecular mechanism linking the ERα-signalling and P-cadherin-regulated expression in breast cancer cell lines. This study showed that ICI 182,780 is able to increase *CDH3* promoter activity, inducing high levels of the active chromatin mark H3 lysine 4 dimethylation. We also observed, for the first time, that the transcription factor C/EBPβ is able to up-regulate *CDH3* promoter activity in breast cancer cells. Moreover, we showed that the expression of P-cadherin and C/EBPβ are highly associated in human breast carcinomas and linked with a worse prognosis of breast cancer patients. This study demonstrates the existence of an epigenetic regulation by which ICI 182,780 up-regulates P-cadherin expression in MCF-7/AZ breast cancer cells through chromatin remodelling at *CDH3* promoter, bringing forward the growing evidence that ERα signalling-abrogation by anti-oestrogens is able to induce the expression of ERα-repressed genes which, in the appropriate cell biology context, may contribute to a breast cancer cell invasion phenotype.**

***CDH3* GenBank accession no. NT_010498.**

INTRODUCTION

Classical cadherins, such as E-, N-, and P-cadherin, are the major structural components of the adherens junctions in many tissues (1). This superfamilly of transmembrane glycoproteins is responsible for calcium-dependent cell–cell adhesion, mediating specific homophilic protein interactions through their extracellular domain and being intracellularly

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linked to the actin cytoskeleton (2). Previous studies showed the involvement of classical cadherins in many biological processes, such as cell recognition, cell signalling, morphogenesis and tumour development (1).

Among these, P-cadherin has extensively been studied by our and other groups, where it has currently been recognized as an important biomarker in breast cancer. In human breast carcinomas, which represent a heterogeneous group of tumours, diverse in behaviour, outcome and response to therapy, P-cadherin was found to be aberrantly expressed in 30–50% of invasive ductal carcinomas of the breast, being strongly associated with proliferative lesions of high histological grade, decreased cell polarity and poor survival of patients over short-term follow-up (3–7). In addition, the expression of P-cadherin, together with other well-described basal markers, such as cytokeratin (CK)5, constitutes one of the most useful adjunctive markers to distinguish basal-like carcinomas of the breast (8).

At the *in vitro* level, our group demonstrated that P-cadherin plays an important role in cell invasion induction through its juxtamembrane domain (5), and that its overexpression induces motility and migration in wild-type E-cadherin breast cancer cell lines, through the secretion of pro-invasive factors, such as matrix metalloproteinase (MMP)-1 and MMP-2. These recent findings revealed the mechanism underlying this *in vitro* invasion behaviour induced by overexpression of P-cadherin and most likely associated with the poor prognosis of breast tumours (9,10).

However, although P-cadherin-associated functions in breast cancer have been extensively studied, the mechanisms controlling P-cadherin overexpression are still unclear.

It is known that the expression of an inappropriate cadherin can result from growth factors and hormones stimulation in the tumour environment, as well as from changes in the promoter regions of cadherin-encoding genes (11). In non-cancer models, *CDH3* promoter was shown to be genetically regulated through direct binding of transcription factors, such as p63 (12) and β-catenin (13).

In 2005, we have reported a significant association between P-cadherin overexpression and the hypomethylation of a specific region of *CDH3* promoter, suggesting an important regulatory role for CpG DNA methylation in the regulation of P-cadherin expression in breast cancer. Interestingly, the study of normal P-cadherin-negative epithelial/luminal cells revealed consistent hypermethylation at this same promoter region (3). The epigenetic regulation of *CDH3*/P-cadherin gene was recently demonstrated in other cancer models, like pancreatic and colorectal carcinomas, as well as in melanomas (14–17).

Indeed, one of our current aims is to find upstream regulators and identify the epigenetic mechanisms that are involved in P-cadherin overexpression in breast cancer cells. In this study, we explored the link between ER-signalling and P-cadherin-regulated expression in breast cancer cell lines, since P-cadherin-positive tumours are essentially ER negative. In fact, our group found that abnormal P-cadherin expression results from a lack of ER-α signalling (5), since treatment of breast cancer cells with the pure anti-oestrogen ICI 182,780 (ICI, Fulvestrant) induced a 2–3-fold increase of P-cadherin protein and *CDH3* mRNA levels in a time- and dose-dependent manner, being this effect counteracted by 17β-oestradiol (E2) (18). Taken together, these previous findings suggested that the lack of ER-α signalling was responsible for the increase of P-cadherin, categorizing *CDH3* as an oestrogen-repressed gene. However, until now, it remained to be determined whether the induction of the *CDH3* gene was due to an epigenetic effect of the anti-oestrogens at *CDH3* promoter level and/or if it would require the prior induction of other genes/proteins.

Herein, we describe the epigenetic remodelling induced by the anti-oestrogen ICI, which leads to higher levels of the active chromatin mark H3 lysine 4 dimethylation (H3K4me2) at *CDH3* promoter sites. We demonstrated in this study that when ER-positive breast cancer cells are treated with ICI, specific transcription sites of the *CDH3* promoter become exposed to putative transcription regulators that, if located nearby, can induce the inappropriate expression of P-cadherin protein. Moreover, we observed, for the first time, that expression of the transcription factor C/EBPβ is able to directly activate P-cadherin promoter and its transcription in breast cancer cells. We further supported our *in vitro* results, showing that the expression of P-cadherin and C/EBPβ are highly associated in human breast carcinomas and linked with a worse prognosis of breast cancer patients.

RESULTS

ERα signalling pathway inhibition induces the transcription and up-regulation of the pro-invasive *CDH3*/P-cadherin in breast cancer cells

P-cadherin expression is tightly regulated by ERα-signalling pathway in breast cancer cells (5). In MCF-7/AZ cells, P-cadherin protein and mRNA expression levels were up-regulated after the treatment with anti-oestrogen ICI and down-regulated by oestradiol (Fig. 1A and B). However, until now, the molecular mechanism leading to increased levels of P-cadherin by ICI was never determined. In this study, we tested whether the ICI-induced P-cadherin overexpression was due to a molecular effect at the *CDH3* promoter level, as a consequence of ERα-signalling pathway deregulation.

To address if ERα-signalling pathway was able to regulate P-cadherin expression levels through *CDH3* promoter activation/repression, a luciferase reporter gene assay was performed in ERα-positive MCF-7/AZ breast cancer cells. The full-length *CDH3* gene promoter was cloned at pGL3-basic vector, as well as *PS2/TFF1* promoter, which is a well-known direct oestrogen-responsive gene, here used as a positive control. Cells were transiently transfected with the pGL3-basic empty vector, *CDH3* or *PS2/TFF1* promoter vector, and treated with E2 or with the anti-oestrogen ICI. As a negative control, cells were treated with ethanol (drug vehicle). As expected, *PS2/TFF1* promoter was strongly activated by E2 and inhibited by the pure anti-oestrogen in the hormonal-dependent MCF-7/AZ breast cancer cells (Fig. 1C). Concerning P-cadherin, we found that ICI significantly increased *CDH3* promoter activity, whereas E2 repressed it (Fig. 1C). These effects mediated by ICI and E2 were detected at mRNA and protein level. Although these differences were not as evident as the ones observed for the positive control *PS2/TFF1* gene, the results were statistically significant. pGL3–control (pLUC) containing a modified coding region for firefly luciferase, optimized for monitoring

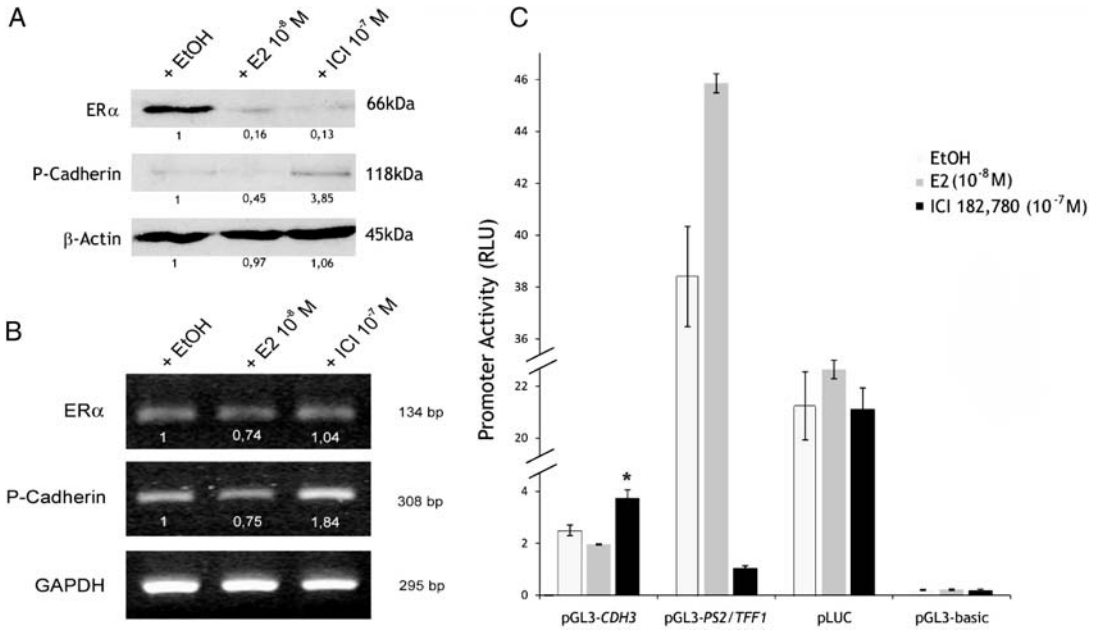

Figure 1. Regulation of P-cadherin expression by the anti-oestrogen ICI 182,780 in MCF-7/AZ breast cancer cells. MCF-7/AZ cells were treated with the indicated concentrations of ICI or 17β-estradiol (E2) for 24 h. (**A**) ERα protein levels of MCF-7/AZ cells treated with E2 or ICI were both decreased, whereas the P-cadherin protein expression was 3.8-fold increased after ICI treatment, relative to the ethanol control treatment. In contrast, P-cadherin expression was reduced in ∼55% when cells were treated with E2. Immunostaining for anti-β-actin was done to control for equal loading. (**B**) mRNA levels of cells treated with E2 or ICI showed no alteration concerning ERα expression, but a marked increased in P-cadherin levels is showed after treatment with ICI. A slight reduction of ∼25% in P-cadherin expression is observed after treatment with E2. GAPDH housekeeping gene amplification was used as a control. (**C**) Luciferase reporter assay quantification was done using relative light units—RLU (relative to *renilla*). ICI significantly induced *CDH3* gene promoter activity when cells were treated with the pure anti-oestrogen ($^{*}P < 0.001$), while E2 slightly induced down-regulation of the promoter activity. Oestrogen-responsive PS2/TFF1 gene promoter vector showed the efficiency and activity of both treatments, namely been activated in the presence of E2 and highly repressed by the presence of ICI. As a negative control, the pGL3-empty vector showed no activity with any of the treatments.

transcriptional activity in transfected eukaryotic cells, was used as positive luciferase assay control. As expected, pLUC– control activity was high and similar in all the treatment conditions. pGL3-basic empty vector did not show any activity with or without treatments.

Similarly, breast cancer cells were transiently transfected with ERα-siRNA, in order to test if the up-regulation of P-cadherin expression could be indeed attributed to specific ERα degradation, or if the ICI-mediated P-cadherin induction could be due to a secondary effect not related to the ERα-signalling pathway. As can be seen in Figure 2A and B, the siRNA for ERα also induced an increased P-cadherin expression at the mRNA and protein level. Overall, we showed that the inhibition of ERα-signalling pathway, by ICI or by ERα-siRNA, induces the transcription and up-regulation of the pro-invasive *CDH3*/P-cadherin in breast cancer cells.

The anti-oestrogen ICI 182,780 up-regulated P-cadherin expression is associated with high levels of the active chromatin mark H3K4me2 at *CDH3* promoter regulatory regions

On the basis of the above-mentioned results, we also aimed to understand the molecular mechanism by which ICI is able to increase the transcription of P-cadherin gene.

Epigenetic mechanisms were already described as induced by ICI (19), which most probably can affect *CDH3* transcription. In previous works, we showed that *CDH3* promoter is able to be regulated by methylation (3), but ICI did not caused any significant change in the methylation pattern of *CDH3* promoter (data not shown). However, it was previously described that pure anti-oestrogen ICI can induce gene transcription through releasing HDACs and ERα from Sp1 sites in ERα-repressed genes (19). Varshochi *et al.* (19) demonstrated that, in the presence of ICI, ERα and HDACs are dissociated from Sp1, resulting in an increased histone acetylation and de-repression of the $p21^{Waf1}$ promoter and expression induction. However, for *CDH3*gene, which promoter is enriched in Sp1 and ERα binding sites, ICI-induced histone acetylation changes were never studied.

In order to access whether *CDH3* promoter is prone to be regulated by acetylation mechanisms, cells transfected with the full-length *CDH3* gene promoter were treated with increased doses of Trichostatin A (TSA), a known histone deacetylase (HDAC) inhibitor. The *CDH3* promoter transfected into MCF-7/AZ breast cancer cells showed a significant dose-dependent activation after treatment with 0.05 μM TSA and with 0.1 μM TSA for 12 h, compared with the activation from the cells treated only with the vehicle [dimethyl sulfoxide (DMSO) (Fig. 3A)]. The increase in *CDH3* promoter activation is also reflected at P-cadherin protein levels (Fig. 3B), indicating that *CDH3* promoter is sensitive to chromatin alterations and that these alterations affect P-cadherin expression. No alterations in ERα expression levels were observed after TSA treatment.

In order to address which type of chromatin modifications could be induced by ICI in *CDH3* promoter potentially

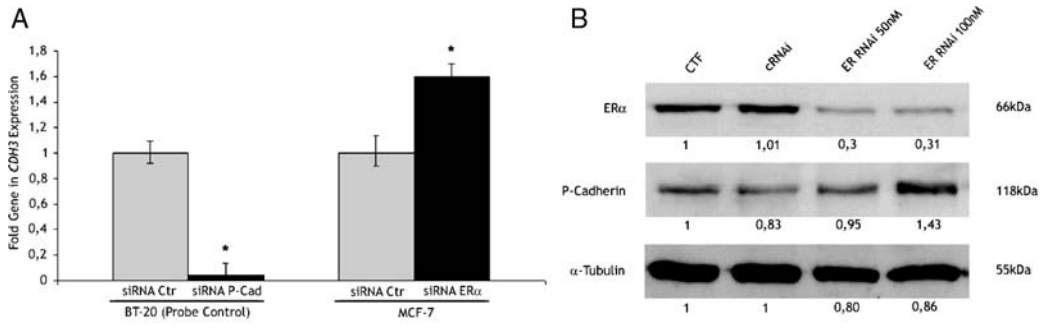

Figure 2. Regulation of P-cadherin expression by the siRNA for ERα in MCF-7/AZ breast cancer cells. (**A**) Real-time PCR showed that cells transfected with siRNA for ERα show a significant increase in P-cadherin expression at the mRNA level. P-cadherin high expressing breast cancer cells BT-20 were used as detection sensitivity control for P-cadherin probe, when P-cadherin is inhibited with a specific siRNA ($^*P < 0.005$). (**B**) At the protein level, the up-regulation of P-cadherin was observed when cells were transfected with an ERα siRNA concentration of 100 nM. The knock-down of ERα expression was observed at both ERα siRNA concentrations of 50 and 100 nM.

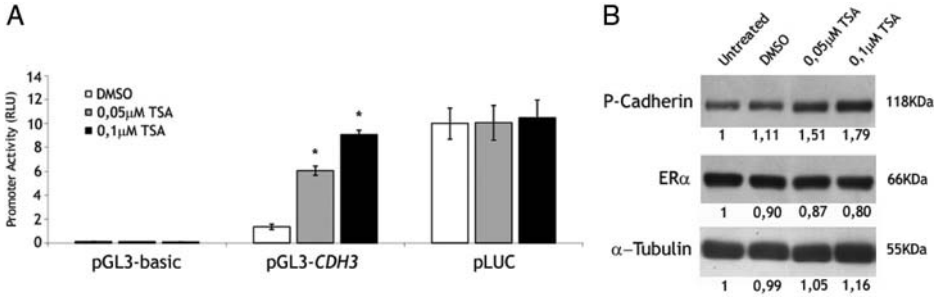

Figure 3. Regulation of *CDH3* promoter and P-cadherin expression by epigenetic activating mechanisms. (**A**) MCF-7/AZ cells transiently transfected with *CDH3* promoter vector were treated with sequential concentrations of TSA during 12 h. An increase of promoter activation was observed when cells were treated with gradual of TSA concentrations ($^*P < 0.0001$). (**B**) The western blot also showed a gradual increased of P-cadherin protein levels without alteration of ERα protein levels.

leading to increased mRNA and protein overexpression, chromatin immunoprecipitation (ChIP) assays were performed using specific antibodies to identify conventional histone-activating (H3K4me2, H3K9ac and H3ac) or repressive marks (H3K27me3 and H4K20me3) within the *CDH3* gene promoter region. We studied two different DNA promoter regions (a distal Region 1 and a proximal Region 2) that were selected according to CpG islands enrichment and to the attributed DNA hypersensitive (DHS) sites within the *CDH3* gene promoter sequence. On the basis of the already described effect of ICI in ERα-repressed genes, predicted ERα and Sp1-binding sites were also considered to select these studied promoter regions (Fig. 4A). No significant alterations in the levels of activating or repressive histones marks were detected at *CDH3* Promoter Region 1 after treatment with ICI (Fig. 4B). However, anti-oestrogen ICI-treatment induced a strong enrichment in H3K4me2 levels, a mark for transcription activation at the proximal *CDH3* promoter Region 2, while neither active H3K9ac and H3ac or repressive H3K27me3 and H4K20me3 marks showed alterations (Fig. 4C).

Altogether, it is suggested that the proximal *CDH3* Promoter Region 2, which is closer to the transcriptional start site (TSS) and ATG, is prone to epigenetic regulation under ICI-treatment, in order to become transcriptionally active.

The C/EBPβ transcription factor activates *CDH3* promoter in breast cancer cells

H3K4me2 is an epigenetic mark which is frequently enriched at regions surrounding known TSSs (20,21). Therefore, we decided to analyse which transcription factors were better represented within a sequence region flanking 250 bp up- and downstream the *CDH3* Promoter Region 2, overlapping with the first nucleotides of the TSS. Combining data from three transcription factors bioinformatic tools (Genomatix, TFSearch and TESS), the transcription factor CCAAT/enhancer-binding protein (C/EBPβ) was predominantly present at this studied region, being this frequency validated by at least two of the three predictive software resources. Accordingly, five putative C/EBPβ-binding sites were found around and comprising the *CDH3* Promoter Region 2 (Fig. 5A), turning this transcription factor as a putative candidate for playing a novel regulatory role on *CDH3* promoter activation in breast cancer cells.

C/EBPβ is a well-known transcription factor and a key regulator of epithelial cell growth, proliferation and differentiation of the mammary gland (22,23). C/EBPβ is expressed in several distinct protein isoforms [liver-enriched transcriptional activating protein (LAP1, LAP2) and liver-enriched transcriptional inhibitory protein (LIP)] that harbour particular regulatory functions (24–26). On the basis of this knowledge,

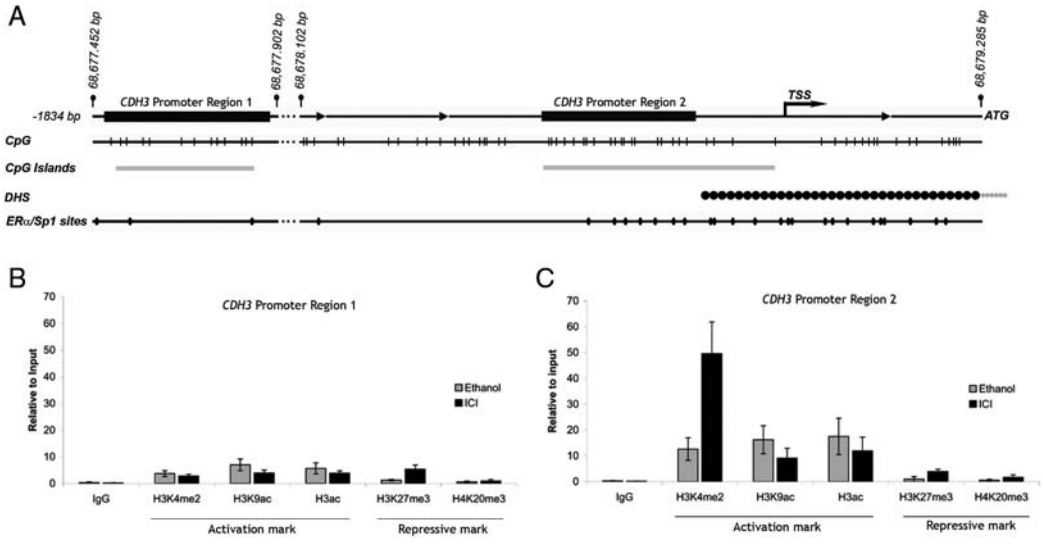

Figure 4. Chromatin immunoprecipitation (ChIP) analysis of histone modifications at *CDH3* promoter after treatment with the pure anti-oestrogen ICI. (**A**) Representation of the *CDH3* promoter structure showing epigenetic regulatory regions (CpG islands and DHS—DNAase hypersensitive sites) and putative ERα/Sp1 sites, predicted by bioinformatic tools (Genomatix, TESS and TFSearch). Transcription start site (TSS) and the analysed distal *CDH3* Promoter Region 1 and proximal *CDH3* Promoter Region 2 are also illustrated. (**B**) and (**C**) ICI-mediated induction of histone activating (H3K4me2, H3K9ac and H3ac) and repressive (H3K27me3 and H4K20me3) marks in *CDH3* Promoter Region 1 and 2, respectively. In promoter Region 1, weak pronounced chromatin alterations were detected after treatment with ICI. In contrast, significant levels of enrichment for H3K4me2 were observed in promoter Region 2, induced by the anti-oestrogen treatment (C).

we decided to test the relevance of these different C/EBPβ isoforms in *CDH3* promoter activation, as well as their relevance in *CDH3* expression at mRNA level.

By luciferase gene reporter assay, using the cloned *CDH3* promoter, different C/EBPβ cDNAs vectors, codifying for a particular C/EBPβ isoform (LAP1, LAP2, or LIP) were independently co-transfected in a cDNA amount titration basis (5, 10 and 20 ng of cDNA) into MCF-7/AZ cells. Luciferase readouts revealed that *CDH3* promoter was gradually activated by the three isoforms, although the promoter activation induced by the C/EBPβ-LIP isoforms was significantly greater compared with the activation induced by LAP1 and LAP2. *CDH3* promoter activation observed with the lowest co-transfected amount of C/EBPβ-LIP (5 ng) was higher when compared with the highest amount of LAP1 and LAP2 isoforms (20 ng) co-transfected into MCF-7/AZ cells. Most importantly, promoter activation was raised when titration was done using higher amounts of C/EBPβ-LIP isoform (Fig. 5B). This result shows that among the three C/EBPβ isoforms with known regulatory functions in breast cancer, *in vitro*, the LIP isoform is the most important in *CDH3* promoter activation.

In order to test this hypothesis, MCF-7/AZ cells were co-transfected with *CDH3* promoter and with C/EBPβ-LIP vector isoform and treated with ICI/control ethanol (EtOH) and luciferase activity was measured. This same experiment was repeated in order to evaluate *CDH3* mRNA expression levels by real-time PCR. The luciferase reporter assay showed that further than the demonstrated activation of *CDH3* promoter by C/EBPβ-LIP isoform, the treatment with ICI provoked a significant synergistic effect towards the activation of *CDH3* promoter (Fig. 5C). The same trend was also observed at the mRNA levels, although not statistically significant (Fig. 5D). In order to see if the induction of P-cadherin expression by ICI would coincide with C/EBPβ nuclear accumulation, immunofluorescence for this transcription factor was performed. Interestingly, we could observe that C/EBPβ is highly expressed at the nuclei of MCF-7/AZ breast cancer cells, independently if these are treated with ICI or EtOH (Fig. 5E).

All these results allowed us to conclude that ICI is able to actively remodel the chromatin at *CDH3* promoter, which permit to expose the C/EBPβ-binding sites. Since C/EBPβ is available in the nuclei of these cells, it will promote P-cadherin transcription and consequent expression.

C/EBPβ is associated with P-cadherin expression and with features of poor prognosis in human breast carcinomas

Similar to what we have previously described for P-cadherin expression, high levels of C/EBPβ have also been associated with tumour progression and as an indicative of an unfavourable prognosis in breast cancer. Most importantly, for the shorter isoform LIP, correlations with ER-negative and poorly differentiated phenotype were previously demonstrated (27,28). Taking into account these results and our *in vitro* data, we decided to perform an immunohistochemical characterization of C/EBPβ expression in a series of 249 invasive primary breast carcinomas previously characterized for P-cadherin expression profile.

From the total 249 cases, only the ones with clear nuclear expression of C/EBPβ were selected for immunohistochemistry classification. Strong immunoexpression of C/EBPβ is observed in the nuclei of luminal epithelial cells from adjacent normal ducts, as showed in Figure 6A. In parallel with what is found for P-cadherin expression in normal epithelial gland (Fig. 6A), C/EBPβ is also expressed in the vast majority of

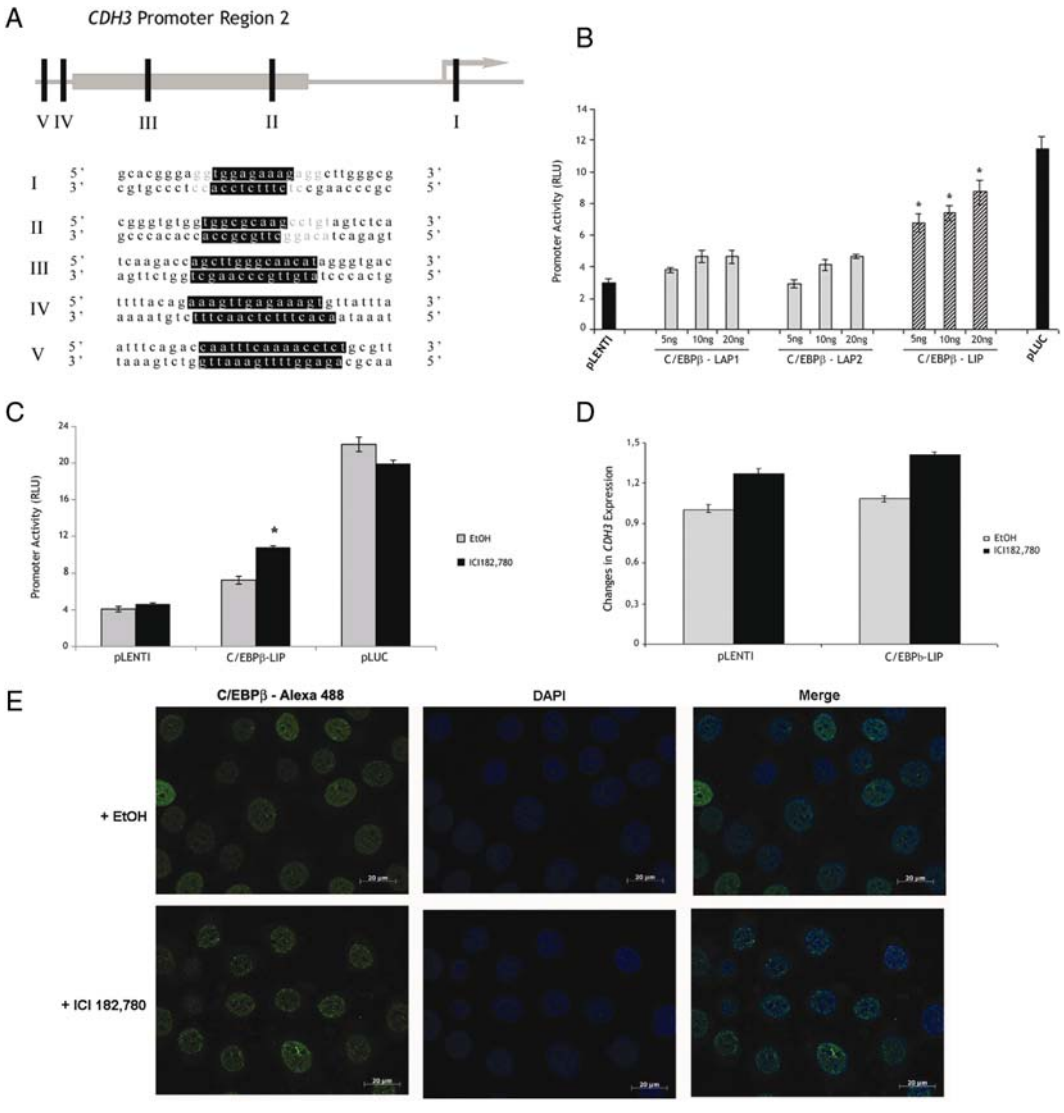

Figure 5. Activation of *CDH3* promoter and P-cadherin mRNA levels by the transcription factor C/EBPβ in MCF-7/AZ cells. (**A**) Proximal *CDH3* promoter region showing five C/EBPβ-binding sites with their predicted sequences based on transcription factors bioinformatic tools. The DNA sequence inside the black area represents the concordant sequence that was validated by at least two of the three bioinformatic tools used (high score), whereas the grey sequence area results from the prediction of a single web tool out of the three used (low score). There is one putative C/EBPβ-binding site at the *CDH3* TSS region and another two inside the studied promoter region 2. Two high-scored C/EBPβ-binding sites are also localized immediately before the limits of the established proximal *CDH3* promoter region. (**B**) *CDH3* luciferase reporter assay where MCF-7/AZ cell were transfected with different amounts (5, 10 and 20 ng) of C/EBPβ cDNA isoforms (LAP1, LAP2 and LIP). Comparative with the pLENTI empty vector, the C/EBPβ−LIP isoform significantly activates the *CDH3* promoter in a dose-dependent manner ($^*P < 0.001$). (**C**) *CDH3* promoter activation by the C/EBPβ−LIP isoform in response to ICI treatment in MCF-7/AZ cells, where the anti-oestrogen induced a synergistic effect with C/EBPβ−LIP towards the activation of *CDH3* promoter ($^*P < 0{,}001$). (**D**) Real-time PCR analysis of P-cadherin mRNA expression levels after ICI treatment. P-cadherin mRNA was up-regulated not only by the transfection of C/EBPβ−LIP isoform, but also by an ICI-mediated synergistic effect. (**E**) MCF-7/AZ cells show nuclear expression of C/EBPβ (Alexa 488—green), treated with ethanol or ICI. The DAPI staining (blue) confirms the nuclei localization of C/EBPβ (see the merge image). All the figures show a ×630 magnification. The white line in each picture represents a 20 μm scale.

the myoepithelial/basal cells. In positive tumour samples, C/EBPβ expression was restricted to the nuclei of malignant cells, while P-cadherin presented its typical membranous staining (Fig. 6B). C/EBPβ was positive (>10% of positive cells) in the nuclei of 43% (86 of 198) of the invasive carcinomas, whereas P-cadherin was present in 33% (64 of 194).

Importantly, C/EBPβ was significantly associated with P-cadherin expression ($P = 0.004$), with nearly 60% of co-expression of these two proteins (Table 1). When we compared the expression of C/EBPβ with the molecular subtype, we found that while 60% of C/EBPβ-negative cases were comprised in the luminal A subtype, basal-like subtype carcinomas expressed C/EBPβ in ∼74% of the cases (Table 1). On the basis of these results, we showed that the expression of C/EBPβ strongly associates with aggressive behaviour features as high proliferation rates, poor differentiation and basal-like phenotype. Furthermore, the expression of C/EBPβ was associated with high histological

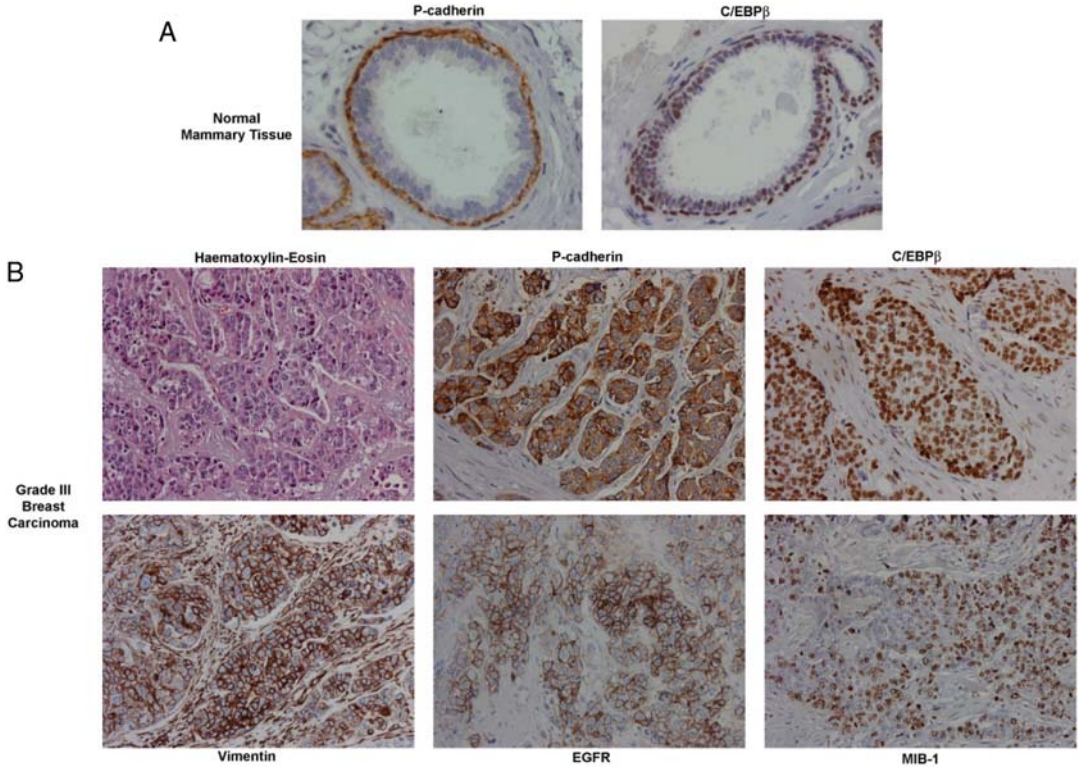

Figure 6. (**A**) Immunohistochemistry representation of the expression pattern of C/EBPβ and P-cadherin in normal breast tissue. P-cadherin expression is restricted to myoepithelial cell layer, whereas nuclear expression of C/EBPβ is seen in myoepithelial cells of a normal breast duct and in scattered normal luminal cells (magnification ×400). (**B**) An immunohistochemical panel of a grade III invasive breast carcinoma (Haematoxylin–eosin staining), showing positive membrane expression for P-cadherin and EGFR, membranar/cytoplasmic expression for vimentin and nuclear expression for C/EBPβ and MIB-1 (magnification ×200).

grade ($P = 0.002$), but no association was found with tumour size or regional lymph node involvement. We also found a strong association of C/EBPβ with breast cancer markers of aggressive phenotype, namely CK14 ($P = 0.015$), vimentin ($P = 0.001$), high proliferative index-MIB-1 ($P < 0.0001$) and EGFR ($P = 0.002$), where 90% of the cases that expressed EGFR were also positive for C/EBPβ. Figure 6B shows an example of a high-grade invasive carcinoma, which was concomitantly positive for P-cadherin, C/EBPβ, vimentin, EGFR and MIB-1 expression.

In summary, we demonstrated, for the first time, an association of the expression of this transcription factor with the expression of P-cadherin, a pro-invasive and migration inducer protein, which also constitutes an important marker of poor prognosis and aggressive basal-like phenotype in breast carcinomas.

DISCUSSION

Over the last years, we and others have been describing the association of P-cadherin expression with malignant behaviour, poor prognosis and short survival in breast cancer (3,6,7,29,30). Recent findings have contributed to the elucidation of P-cadherin function in breast tumour cell biology (10), but the expression regulation of this protein in breast cancer has poorly been explored. Previously, we reported a significant association between P-cadherin overexpression and *CDH3* hypomethylation, suggesting an important regulatory role of epigenetic events in the regulation of P-cadherin expression in breast cancer (3). Moreover, we demonstrated that the abrogation of ERα-signalling pathway, caused by the pure anti-oestrogen ICI, was responsible for the increase of P-cadherin protein and mRNA expression, pointing *CDH3* as an oestrogen-repressed gene (5). However, the mechanisms by which epigenetic events and ERα-signalling inhibition leads to P-cadherin expression and to aggressive tumour behaviour are still unknown.

In the present study, we identified, for the first time, the existence of an epigenetic regulation by which ICI up-regulates P-cadherin expression in MCF-7/AZ breast cancer cells through chromatin remodelling at *CDH3* promoter. After treatment of this ERα-positive breast cancer cell line with the oestrogen antagonist ICI, an important histone-activating mark (H3K4me2) was enriched at the proximal region of the *CDH3*/P-cadherin promoter.

Previous studies showed that chromatin structural remodelling and nuclear entropy can be induced by the treatment of breast cells with anti-oestrogens such as ICI (31). In fact, and although it was initially believed that anti-oestrogens function merely by competing with endogenous oestrogens for receptor binding, recent studies also demonstrated that ICI and tamoxifen can induce distinct conformational changes in ER, implying that the ligand-bound ER can recruit specific co-regulators to modulate different gene promoters, thereby regulating gene expression (32,33).

Table 1. Association of C/EBPβ expression with clinicopathological features and immunohistochemical markers in invasive breast carcinomas

Variables	No.	C/EBPβ negative (%)	C/EBPβ positive (%)	*P*-value
Tumour size	187			
<15 mm	16	11 (68.7)	5 (31.3)	0.288
≥15 mm	171	94 (55.0)	77 (45.0)	
LNI	180			
Present	91	55 (60.4)	36 (39.6)	0.138
Absent	89	44 (49.4)	45 (50.6)	
Tumour grade	198			
GI	42	31 (73.8)	11 (26.2)	0.002
GII	90	54 (60.0)	36 (40.0)	
GIII	66	27 (40.9)	39 (59.1)	
NPI	169			
NPI < 3.4	38	26 (68.4)	12 (31.6)	0.067
3.4 ≤ NPI ≤ 5.4	85	39 (45.9)	46 (54.1)	
NPI > 5.4	46	25 (54.3)	21 (45.6)	
EGFR	197			
Positive	10	1 (10.0)	9 (90.0)	0.002
Negative	187	111 (59.4)	76 (40.6)	
P-Cadherin	194			
Positive	64	27 (42.2)	37 (57.8)	0.004
Negative	130	83 (63.8)	47 (36.2)	
CK5	198			
Positive	41	22 (53.7)	19 (46.3)	0.673
Negative	157	90 (57.3)	67 (42.7)	
CK14	193			
Positive	10	2 (20.0)	8 (80.0)	0.015
Negative	183	107 (58.5)	76 (41.5)	
Vimentin	178			
Positive	25	7 (28.0)	18 (72.0)	0.001
Negative	153	95 (62.0)	58 (38.0)	
MIB-1	197			
<10	94	70 (74.5)	24 (25.5)	<0.0001
10–20	20	9 (45.0)	11 (55.0)	
>20	83	33 (39.8)	50 (60.2)	
Subtype	178			
Luminal	122	74 (60.7)	48 (39.3)	0.001
HER-2	22	14 (63.7)	8 (36.3)	
Basal	34	9 (26.5)	25 (73.5)	

Data presented as n (%) unless stated otherwise.
C/EBPβ, CCAAT/enhancer-binding protein beta; LNI, lymph node involvement; NPI, Nottingham prognostic index; EGFR, epidermal growth receptor; P-cadherin, placental cadherin; CK, cytokeratin; MIB-1, mindbomb homolog 1; HER-2, human epidermal growth factor receptor 2.

A study in 2005 showed that ICI can induce transcription of the ERα-repressed gene $p21^{Waf1}$, through the dissociation of HDACs and ERα from Sp1 sites and therefore, resulting in increased histone acetylation and de-repression of the $p21^{Waf1}$ promoter (19). In fact, the authors not only found that the proximal Sp1 sites are crucial in mediating the promoter's response to ICI, but also that HDAC inhibition by TSA leads to $p21^{Waf1}$ promoter activity (19). Further than the fact that most of the proximal promoter regions are generally important to gene transcription regulation, the studied *CDH3* proximal Region 2, which showed an ICI-induced enrichment for the active histone mark H3K4me2, additionally displays a CpG island and a DNaseI hypersensitive site (DHS) region, overlapping with the TSS. As described by the ENCODE Project Consortium, the aggregate signal of histone modifications is mainly attributable to active TSS region, in particular those near CpG islands and DHS, both genomic regions thought to be enriched for regulatory information (34). Importantly, after a prediction analysis of the proximal *CDH3* promoter, comprising a region from the TSS to the ATG, we observed that this promoter area was remarkably enriched in Sp1 sites, having also a significant number of ERα coupled with those (Fig. 4A). Hence, if a repression complex, mediated by ERα and HDACs at Sp1 sites, is able to be released by the treatment with ICI and therefore enhancing the gene transcription, the characteristics of the proximal *CDH3* promoter, together with the H3K4me2 enrichment in Region 2, strongly suggest that this chromatin de-repression mechanism plays an important role in the ICI-induced promoter transcriptional activation. Reinforcing this, we also observed an up-regulation of *CDH3* promoter activity and P-cadherin protein expression in cells treated with TSA, showing that chromatin-activating modifications are indeed important to the modulation of this gene.

The most prominent activating mark found within the *CDH3* promoter Region 2 was H3K4me2, which is the histone modification better correlated with DHS regions and chromatin accessibility (20,21,34), as well as with active gene transcription (35).

Herein, we further investigated which transcription factors were strongly represented within *CDH3* promoter Region 2, TSS and DHS region. We have found that putative C/EBPβ-binding sites were predominantly present within this region. Furthermore, we tested the ability of C/EBPβ to transactivate P-cadherin protein and mRNA expression, as well as *CDH3* promoter, demonstrating, for the first time, that, among the three different C/EBPβ isoforms, C/EBPβ-LIP was the most relevant in a P-cadherin expression activation setting. C/EBPβ proteins are transcription factors which regulate cellular proliferation, differentiation and apoptosis in mammary gland (24). However, like P-cadherin, C/EBPβ is not mutated in breast tumours, but its overexpression has widely been described in a subset of aggressive breast cancer (25). Interestingly, transgenic and overexpression studies showed that C/EBPβ–LIP induces proliferation in mammary epithelial cells and that a C/EBPβ–LIP-initiated growth cascade may play a role in the development of breast cancer (24,26). At a clinicopathological level, LIP isoform correlates with an ER-negative breast cancer phenotype, high proliferative index and histological grade, aneuploidy and poor differentiation. These findings are not only suggestive of the involvement of C/EBPβ–LIP in tumour progression and indicative of an unfavourable patient prognosis (27), but also show that its expression should be evaluated as a prognostic marker for breast cancer patients (28). Remarkably, breast carcinomas expressing C/EBPβ–LIP displays the unfavourable clinicopathological features described for the aggressive breast tumours overexpressing P-cadherin. Additionally, we also observed an association of C/EBPβ with aggressive markers, namely EGFR, CK14 and vimentin expression, as well with basal-like molecular phenotype. Thus, based in our results, it is tempting to consider that, under conditions of ICI-mediated increased chromatin accessibility, C/EBPβ, and most likely the LIP isoform, can play a role in the activation of *CDH3* promoter towards a typical P-cadherin-related aggressive tumour phenotype.

Our results are also of clinical relevance since there is growing evidence that selective ER modulators, such as

tamoxifen or ICI (Fulvestrant), are able to induce expression of genes which, in the appropriate cell context, may contribute to adverse cell phenotype, in part by inducing breast cancer cell invasion (36). Although anti-oestrogens have been the mainstay of therapy in patients with ERα-positive breast cancer and have provided significant improvements in survival, their benefits are limited by tumour recurrence in a significant proportion of initially drug-responsive breast cancer patients due to acquired anti-oestrogen resistance (36). Therefore, it is tempting to assume that one of the important mechanisms by which this endocrine resistance occur should be the inappropriate activation of ER-repressed genes at late stages of long-course endocrine therapeutic regimens.

To date, mechanistic studies have revealed important roles for growth factor signalling pathways, such as those regulated EGFR and HER2, as contributors to endocrine resistance (37–39). Similarly, in ER-positive breast cancer cells, tamoxifen has been reported to increase the expression of poor prognosis markers in breast cancer patients [14-3-3σ (40)], as well as of signalling elements frequently linked to tumour migration and invasion (MAPK, FAK and Src) (41,42).

The present study highlights that, in ERα-positive breast cancer cells, the anti-oestrogen ICI is able to induce the expression of *CDH3* gene, leading to P-cadherin overexpression, which is described as a pro-invasive protein in breast cancer. These data, together with other studies, contribute to clarify the ability of selective ER modulators and steroidal anti-oestrogens, like fulvestrant, to induce expression of genes normally repressed by oestrogen/ER signalling, and thus, playing an important role in the capacity of breast cancer cells to evade their growth inhibitory effects (37,43). It is important to know which signalling pathways are activated in anti-oestrogen resistant breast cancer, in order to find new and effective therapeutic targets to use in this setting. In the future, it would be interesting to study if P-cadherin can be a good biomarker in this group of breast tumour recurrences that occur upon endocrine therapy.

MATERIALS AND METHODS

Antibodies and chemicals

The following primary anti-human antibodies were used for western blot (WB), immunohistochemistry (IHC) and immunofluorescence (IF) against: P-cadherin [mouse monoclonal, clone 56; BD Transduction Biosciences, Lexington, KY, USA; dilutions: 1:250 (WB) and 1:50 (IHC)], C/EBPβ [mouse monoclonal, clone H7, Santa Cruz Biotechnologies, CA, USA; dilutions: 1:500 (WB) and 1:100 (IHC, IF)], β-actin [goat monoclonal, I-19; Santa Cruz Biotechnologies; dilutions: 1:1000 (WB)], ERα [mouse monoclonal, NCL-L-ER-6F11; Novocastra, Newcastle; dilutions: 1:50 (WB) and 1:200 (IHC)]. Anti-mouse and anti-goat horseradish peroxidase-conjugated secondary antibodies were also used for WB [HRP-conjungated, Santa Cruz Biotechnologies; dilutions: 1:2000]. For chromatin immnunoprecipitation (ChIP) assays, the following antibodies were used: anti-acetyl-H3K9 antibody (07–352; Upstate Biotechnology, Lake Placid, NY, USA), anti-acetyl-H3 antibody (17–615; Upstate), anti-dimethyl-H3K4 antibody (07–030; Upstate), anti-trimethyl-H3K27 antibody (07–449; Upstate), anti-trimethyl-H4K20 antibody (ab9053; Abcam plc, Cambridge, UK) and rabbit anti-mouse-IgG antiserum (M7023; Sigma-Aldrich, Bornem, Germany).

ICI 182,780 Imperial Chemical Industries (ICI), 17β-oestradiol (E2) and Trichostatin A (TSA) were all purchased from Sigma. ICI and E2 were dissolved in 100% ethanol (EtOH) while TSA was dissolved in DMSO.

Cell culture, transfection and treatment conditions

The human breast cancer cell line MCF7/AZ was kindly given by Prof. Marc Mareel (Laboratory of Experimental Cancerology, Ghent University, Belgium), whereas BT-20 breast cancer cell line was purchased from American Type Culture Collection—ATCC (Manassas, VA, USA). Cell lines were cultured in growth media consisting of Dulbecco's modified Eagle's medium [DMEM (Invitrogen Ltd, Paisley, UK)], supplemented with 10% heat inactivated fetal bovine serum (Invitrogen), 100 IU/mL penicillin and 100 μg/ml of streptomycin (Invitrogen), at 37°C. MCF/AZ cells were grown at 10% CO_2 and BT-20 cells at 5% CO_2 controlled atmosphere.

For transient transfections, reagents were used as described subsequently. For gene reporter assays, MCF-7/AZ cells were grown in 96-well plates to 60–70% confluence and transfection was achieved using the liposome-mediated FuGENE 6 transfection reagent (Roche Diagnostic GmbH, Mannheim, Germany), prepared according to the manufacturer's instructions. These transient transfections used a charge ratio (FuGENE/DNA) of 3:1 where equal amounts (20 ng) of *CDH3* promoter vector, as well as from the expression vector, were added together with 5 ng of pCMV-Renilla normalization vector. For RNA or protein expression assays, MCF-7/AZ cells were grown in 6-well plates to 70–80% confluence. Transient transfections of 1 μg of C/EBPβ expression vector were done using Lipofectamine 2000 (Invitrogen), using a ratio (Lipofectamine/DNA) of 3:1 prepared according to the manufacture's instruction.

Whenever not specified, cells treatments with ICI were carried out for 24 h at a final concentration of 0.1 μM (10^{-7} M), while treatments with E2 were performed at a final concentration of 0.01 μM (10^{-8} M) for 24 h. In treatments with TSA, cell measurements were done after 12 h of incubation at the final concentration of 0.05 μM or 0.1 μM.

Promoter vectors and cDNA constructs

The human pLENTI-C/EBPβ expression vectors (C/EBPβ-LAP1, C/EBPβ-LAP2 and C/EBPβ-LIP) were kindly provided by Dr. Peter Gott (Institute of Anthropology and Human Genetics, Tübingen, Germany). To generate the full-length *CDH3*-luciferase vector, a 2.1 kb 5′ untranslated region of human *CDH3* gene (GenBank accession no. NT_010498) was generated by PCR, using a *Pfu* DNA Polymerase (MBI Fermentas, Burlington, Canada), the sense primer (5′-TGCTAGGCCTGAGAGAGCAAG-3′) and antisense primer (5′-CCTTCCGGGACTCCCTTG-3′). The PCR product was subcloned into a TOPO Cloning TA vector (Invitrogen) and then transferred to a pGL3-luciferase reporter plasmid (Promega, Corporation, Madison, WI, USA), after

digestion of both recipient vector and PCR fragment with *Kpn*I and *Nco*I restriction enzymes (MBI Fermentas). Ligation was performed using T4 ligase enzyme (New England Biolabs, Ipswich, MA, USA), and a pGL3/*CDH3*-luciferase reporter full-length vector (positions from −1834 to +1 ATG site), framed with ATG/firefly luciferase cDNA from the pGL3-luciferase reporter plasmid, was generated. Direct sequencing (ABI, Perkin-Elmer, Foster City, CA, USA) was performed to confirm the cloning frame and integrity of the promoter.

CDH3-luciferase reporter gene assay

MCF-7/AZ cells were co-transfected with the human full-length pGL3-*CDH3*/*luc* promoter vector and with pCMV-Renilla luciferase construct (Promega), for normalization of transfection efficiency. For promoter analysis, 24 h after transfection, cells were washed twice in PBS-cold and then harvested and lysed for firefly/Renilla luciferase assays using the Luclite Reporter Gene Assay System (Perkin Elmer, Beaconsfield, UK), according to the manufacturer's instructions. Luciferase bioluminescence from Renilla was measured using native coelenteranzine substrate reagent (Lux Biotechnology, Edinburgh, UK). Individual transfection experiments were repeated at least three times and in quadruplicate per transfection condition. Empty pGL3-basic vector (E1751) and pGL3-Control (pLUC) vector (E1741), both from Promega, were included as controls in all *CDH3*-reporter assays. Luminescence was then read using the Wallac/Perkin Elmer-1450-028 Trilux Microbeta (Perkin Elmer) plate reader, and the results are shown as mean of relative light units (RLU).

Protein extraction and WB analysis

Protein lysates were prepared from cultured cells, using catenin lysis buffer [1% (v/v) Triton X-100 and 1% (v/v) NP-40 (Sigma) in deionized phosphate-buffered saline (PBS)] supplemented with 1:7 proteases inhibitors cocktail (Roche Diagnostics GmbH, Germany). Cells were washed twice with PBS and were allowed to lyse in 500 µl of catenin lysis buffer for 10 min, at 4°C. Cell lysates were mixed with a vortex for three times and centrifuged at 20 000*g* at 4°C, during 10 min. Supernatants were collected and protein concentration was determined using the Bradford assay (BioRad Protein Assay kit). Proteins were dissolved in sample buffer [Laemmli with 5% (v/v) 2-β-mercaptoethanol and 5% (v/v) bromophenol blue] and boiled for 5 min at 95°C. Samples were separated by SDS–PAGE, and proteins were transferred into nitrocellulose membranes [Amersham Hybond enhanced chemiluminescence (ECL)] at 130 V for 1 h. For immunostaining, membranes were blocked with 5% (w/v) non-fat dry milk in PBS containing 0.5% (v/v) Tween-20. These were subsequently incubated with primary antibodies, during ∼1–2 h, followed by four 5 min washes in PBS/Tween-20 (PBS-T). Then the membranes are incubated with horseradish peroxidase-conjugated secondary antibodies, during 45 min. Proteins were detected using ECL reagent (Amersham), as a substrate, and blots were exposed to an autoradiographic film. Quantification of WBs was performed using Quantity One software (BioRad), and the ones selected to show are representative experiments.

Immunofluorescence

Briefly, MCF-7/AZ cells were cultured on glass coverslips, and fixed with cold methanol for 10 min on ice. After fixation, cells were permeabilized with 1% Triton X-100 in PBS for 5 min, at room temperature. Non-specific binding was blocked by cell treatment with PBS containing 3% BSA, for 30 min, at room temperature. Cells were then stained with the primary antibody for C/EBPβ (Santa Cruz), during 1 h, at 1:100 dilution. After PBS washes, cells were incubated, for 1 h, with the rabbit polyclonal secondary antibody, conjugated with Alexa 488 (Invitrogen), at 1:500 dilution. After a wash with PBS, each sample was mounted with Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA) containing 4,6-diamidine-2-phenylindolendihydrochrolide (DAPI). The C/EBPβ and DAPI dual cell staining was observed with a Zeiss microscope (Imager Z1) with apotome, and images were acquired using the Axiovision software.

ChIP assay

Exponentially growing MCF-7/AZ cells were treated with ICI for 24 h and fixed with 1% formaldehyde (37°C, 10 min). The reaction was stopped with addition of glycine to a final concentration of 0.125 M. Whole-cell fixed lysed extracts were prepared for use in ChIP assays as described previously (44). Briefly, fragmented chromatin to an average size of 300–1000 bp was incubated (4°C, 1 h) with 30 µl of blocked protein-A-agarose beads (Sigma) on a rotating wheel. Pre-cleared chromatin (150 µg) was immunoprecipitated (4°C, overnight) with 10 µl anti-acetyl-H3K9 antibody, 10 µl anti-acetyl-H3 antibody, 5 µl anti-dimethyl-H3K4 antibody, 5 µl anti-trimethyl-H3K27 antibody, 5 µl anti-trimethyl-H4K20 antibody and 2 µl of a rabbit anti-mouse-IgG antiserum as a negative control. After elution of immune complexes, DNA was resuspended in 100 µl of TE (10 mM Tris, 1 mM EDTA at pH 8.0) solution. Quantification of precipitated DNA was performed using real-time qPCR amplification carried out on a Chromo4 DNA engine (Biorad), using SYBR green jumpstart PCR master mix (Sigma) and 0.3 µM of the following primers: distal promoter region 1, forward: 5′-CAGGTTAGCCCTGGAAGGTCAA-3′; reverse: 5′-TGAGATGGAGTCTCACTGTCGTCC-3′; proximal promoter region 2, forward 5′-CTGTGAAATGGAAG AAGCGGTC-3′, reverse 5′-GCTGGTCTTGAACTTCTGGA CTC-3′. The amount of DNA precipitated by each antibody was normalized against 1 in 10 of the starting input material.

Gene silencing with small interfering RNAs (siRNAs)

MCF-7/AZ and BT-20 cells in 2 ml of culture medium were transfected with siRNA for ERα (50 nM and 100 nM Hs_ESR1, GW Validated siRNA, Qiagen, Cambridge, MA, USA) or for P-Cadherin (50 nM, Hs_CDH3_6, GW Validated siRNA, Qiagen). Transfection was carried out at starvation conditions for 6 h after which appropriated culture, medium was added to the cells. After 48 h, the cells were harvested

for RNA isolation or protein extraction for real-time PCR or WB analysis.

RNA isolation and real-time PCR

RNA was isolated using a Qiagen RNAeasy extraction kit (Qiagen), according to the protocol provided by the manufacturers and concentration was determined in a ND-1000 spectrometer (Nanodrop). One microgram of RNA per sample was used to synthesize cDNA, using reverse-transcriptase RT (Invitrogen). *CDH3* TaqMan probe (HS00354998_m1, Applied Biosystems, Foster City, CA, USA) was used to specifically recognize *CDH3* fragments, which were amplified through 40 cycles (Applied Biosystems 7000). Relative *CDH3* gene expression was determined by its normalization with *GAPDH* expression, using a Human *GAPDH* endogenous control (NM_002046.3, Applied Biosystems).

Patient selection

A series of 249 cases of primary operable invasive breast carcinomas were retrieved from the files of the Department of Pathology, Hospital of Divino Espírito Santo, Azores, Portugal and from the Federal University of Santa Catarina, Florianópolis-SC, Brazil. These samples were obtained from patients with age ranging from 30 to 89 years old. All the formalin-fixed paraffin-embedded histological sections were reviewed by three pathologists (V.C., F.S. and F.M.) and the diagnoses were confirmed as follows: 208 invasive ductal carcinomas, 7 invasive lobular carcinomas, 3 mixed, 3 tubular, 8 medullary and 20 invasive breast carcinomas of other special histological types. These tumours have been fully characterized for the clinical and pathological features, namely tumour size, lymph nodes invasion, tumour grade, Nottingham Prognostic Index (NPI) and for the following breast cancer markers: ERα, PR, HER-2, EGFR, P-cadherin, CK5, CK14, vimentin and MIB-1, as well as classified for breast cancer subtype (4,7,8,45,46).

This study was conducted under the national regulative law for the usage of human biological specimens, where the samples are delinked from their donor's identification and are exclusively available for retrospective research purposes.

Immunohistochemistry analysis

IHC was performed in 3 μm formalin-fixed paraffin-embedded sections. The IHC technique was performed using an Envision Detection System (DAKO Cytomation Envision System HRP, DAKO Corporation, Carpinteria, CA, USA) or the classical streptavidin–avidin–biotin complex (SABC) method according with the manufacturer's instructions.

Expression of C/EBPβ was analysed using a mouse monoclonal antibody. Sections were deparaffinised with xylene and rehydrated in a series of decreasing concentrations of ethanol solutions. Heat-induced epitope retrieval was carried out in 10 mM citrate buffer (sodium citrate) (pH 6) (LabVision Corporation, Fremont, CA, USA), in a 98°C water bath, for 30 min. After cooling retrieval solutions, for at least 30 min at room temperature, the slides were treated for 10 min with 3% H_2O_2 in methanol, in order to block endogenous peroxidase. Slides were incubated overnight at 4°C with monoclonal antibody for C/EBPβ and then labelled with the Envision Detection System from DAKO. Colour reaction product was developed with 3,3′-diaminobenzidine, tetrahydrochloride (DAB plus, DAKO Glostrup, Denmark) as a substrate, and nuclear contrast was achieved with haematoxylin/ammoniacal water counterstaining. Formalin-fixed paraffin-embedded sections from normal breast gland, skin or normal gastric mucosa were used as positive controls. Also, negative controls were performed by replacing the primary antibody with PBS/non-immune mouse serum.

Immunostained slides were reviewed by two pathologists (F.M. and F.S.) and cases with >10% of nuclei-stained positive cells for C/EBPβ were considered positive.

Statistical analysis

C/EBPβ immunoexpression associations were analysed using StatView, version 5.0 software (SAS Institute, Inc.). Continuous variables were presented as mean ± standard deviation (SD), and categorical variables were presented as number (%). The clinicopathological features and immunohistochemical markers of the tumours were compared across groups of expression of C/EBPβ using ANOVA and the chi-square test, respectively, for continuous and categorical variables.

For luciferase reporter gene analysis, independent quadruplicate measurements per each analysed variable were performed and RLU were compared between variables using Student's *t*-test. This same statistical method was also used to assess the variations in RNA expression.

WEB RESOURCES

URLs for *CDH3*-related data presented in this article and accession numbers used were the following:

(1) GenBank, http://ncbi.nlm.nih.gov/GenBank, NM_001793.
(2) Ensemble,http://www.ensembl.org/Homo_sapiens/Location/Overview?r=16:68677452-68679285
(3) New England Biolabs Cutter, http://tools.neb.com/NEBcutter2/index.php.
(4) Transcription Element Search System (TESS), http://www.cbil.upenn.edu/cgi-bin/tess/tess.
(5) CpG Island Searcher, http://cpgislands.usc.edu/cpg
(6) TFSEARCH, http://www.cbrc.jp/research/db/TFSEARCH.html.
(7) Genomatix, http://www.genomatix.de.

ACKNOWLEDGEMENTS

The authors would like to thank to Catarina Pereira by the effort and dedication in retrieving and organizing the breast cancer samples for this work.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by research grants from FCT—Portuguese Foundation for Science and Technology [grant

numbers SFRH/BD/15316/2005 to A.A. and SFRH/BD/36096/2007 to A.S.R.]. The work was mainly supported by a scientific project (POCI/BIA-BCM/59252/2004) funded by FCT. IPATIMUP is an Associate Laboratory of the Portuguese Ministry of Science, Technology and Higher Education and is partially supported by FCT.

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PUBLICATIONS

Paper 5

Histol Histopathol (2010) 25: 963-974

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

P-cadherin, Vimentin and CK14 for identification of basal-like phenotype in breast carcinomas: an immunohistochemical study

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Summary. Introduction: The most suitable immunohistochemical criterion to identify basal-like breast carcinomas (BLBC), a molecular subgroup of breast cancer associated with poor prognosis, is the triple negative phenotype along with CK5 and/or EGFR immunoreactivity. However, several putative basal markers have been suggested as alternatives to identify BLBC with more accuracy. Experimental Design: The expression of CK5, EGFR, P-cadherin, CK14, Vimentin and p63 were evaluated in 462 invasive breast carcinomas to determine their sensitivity and specificity for BLBC identification. Results: P-cadherin and CK5 showed higher sensitivity values, while EGFR, Vimentin and CK14 were the most specific markers. The combination of CK5 with P-cadherin, Vimentin or CK14 proved to be a reliable option for distinguishing the basal phenotype, compared to the "gold standard" pair CK5/EGFR. Furthermore, P-cadherin was still able to recognize a large number of putative BLBC among the "unclassified" group (ER-/PR-/HER2-/CK5-/EGFR-). Conclusions: P-cadherin, Vimentin and CK14 can recognize BLBC already identified in triple negative/ CK5 and/or EGFR+ tumors, and due to P-cadherin sensitivity for BLBC identification this marker can reliably recruit a large number of breast carcinomas with basal phenotype among immunohistochemistry triple negative/ CK5 and/or EGFR - pool of tumors. Although they need GEP validation, our results can introduce the idea of these markers as additional options in the daily workup of breast pathology laboratories to identify BLBC.

Key words: Basal-like breast cancer, P-cadherin, CK14, Vimentin

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Introduction

In the European Union, breast cancer is the most incident form of cancer in women, with an estimated 429.900 cases diagnosed per year (28.9% of all incident cases in women) (Ferlay et al., 2007; Milanezi et al., 2008). Breast cancer is frequently designated as a heterogeneous disease with divergent biological behaviors. cDNA microarray studies have provided an improvement in cellular and molecular understanding of breast cancer, identifying distinct subtypes of breast carcinomas with different molecular signatures and clinical outcomes (Perou et al., 2000; Sorlie et al., 2001, 2003; Rakha et al., 2006a,b). The basal-like subtype has definitely drawn the attention of the scientific community. These tumors are characterized by a triple negative (TN) phenotype, lacking the expression of hormone receptors (HR) [estrogen and progesterone receptors (ER and PR, respectively)] and HER2. Basal-like breast carcinomas (BLBC) are associated with

aggressive tumor behavior and shorter overall survival when compared to the luminal and HER2-overexpressing subtypes and there is an enthusiastic search for molecular markers expressed in BLBC that could be used as targets to therapy (Nielsen et al., 2004). Histologically, they are poorly differentiated carcinomas, present high nuclear and histological grade and frequently show medullary and metaplastic features (Tsuda et al., 2000; Fulford et al., 2006; Livasy et al., 2006; Rakha et al., 2006a,b). A distinct pattern of metastasis to brain and lungs, known to be associated with poor prognosis, and less significant involvement of axillary lymph nodes, has also been described in BLBC (Tsuda et al., 2000; Banerjee et al., 2006; Fulford et al., 2007). Nowadays, gene expression profiles (GEP) or cDNA microarrays studies are currently considered the "gold standard" methods for the identification of breast carcinomas with basal phenotype, since these technologies were the first to identify BLBC as a distinct subgroup with a specific molecular signature (Perou et al., 2000) and clinical identity (Sorlie et al., 2001, 2003; van't Veer et al., 2002). However, GEP are expensive, not easily applicable as a routine laboratory diagnostic tool in large scale clinical-pathological analysis and have limited value in retrospective studies using formalin-fixed paraffin-embedded (FFPE) tissues (Cheang et al., 2008; Reis-Filho and Tutt, 2008). Thus, the idea of developing an immunohistochemical (IHC)-based assay for the identification of BLBC is appealing. The variation in the transcriptional and translational programs of cells that accounts for the different molecular identities of breast carcinomas also reinforces the interest in creating an IHC-based assay for BLBC definition. The characteristic protein expression of tumors would be a useful surrogate of GEP, and the IHC profile would help to standardize investigations and uniformly identify a group of tumors with a basal-like transcriptional program (Reis-Filho and Tutt, 2008).

However, the most appropriate panel of antibodies to be used, in order to identify breast carcinomas with basal phenotype, has not reached a consensus yet. In 2008, Tang et al. (2008) compared the different IHC classifications that have been used to define basal-like and non basal-like breast carcinomas; interestingly, they showed that in high grade breast carcinomas, which is a common feature of basal phenotype, the rates of BLBC ranged between 19% and 76%, indicating the need for a more consensual strategy between laboratories.

The TN phenotype criterion is used by some authors who assume that Triple Negative tumors and BLBC are synonymous (Kreike et al., 2007; Spitale et al., 2008). In fact, this criterion is quite convenient, since it includes standard biomarkers already used in the clinical management of breast cancer. However, relying on negative results to perform a diagnostic interpretation may be risky due to technical failures leading to a decrease in specificity. Other authors use high molecular weight cytokeratins alone (CK5/6, CK14 or CK17) to identify BLBC, claiming that BLBC and triple negative tumors are different identities (van de Rijn et al., 2002; Abd El-Rehim et al., 2004; Fulford et al., 2007; Rakha et al., 2007b). In addition, since basal-like breast carcinomas express proteins that are characteristic from the basal/myoepihelial outer layer of the mammary gland, such as EGFR, p63, P-cadherin, calponin, CD10, S100 and α-smooth-muscle actin (α-SMA) (Jones et al., 2001; Reis-Filho et al., 2003; Nielsen et al., 2004; Livasy et al., 2006), some definitions of BLBC associate the lack of expression of ER, PR and HER2 with the immunoreactivity for some of these basal markers that were already correlated with basal phenotype and poor prognosis (Nielsen et al., 2004; Matos et al., 2005; Laakso et al., 2006). Our group has previously demonstrated that using a panel of antibodies for ER, PR, HER2, CK5/6 and/or EGFR and/or P-cadherin and/or p63 it is possible to distinguish invasive (Matos et al., 2005) and i*n situ* (Paredes et al., 2007b) BLBC. However, Nielsen et al. (2004) found that expression of CK5/6 and EGFR together with negativity for ER and HER2 would be the immunoprofile that identifies the same basal-like carcinomas found by cDNA microarrays, with a sensitivity of 76% and a specificity of 100%. This criterion is, therefore, considered the "gold standard" immunoprofile to classify BLBC.

In this study, we aim to refine the immunohistochemical criterion to identify BLBC by analyzing the sensitivity and the specificity of the main basal markers that have been described, namely CK5, EGFR, P-cadherin, CK14, Vimentin and p63 and suggest possible additional markers for BLBC identification, especially in CK5 and EGFR negative breast carcinomas.

Materials and methods

Breast tumour samples

Formalin-fixed, paraffin-embedded tissues of 462 invasive breast carcinomas were consecutively retrieved from the histopathology files of three Departments of Pathology: University Hospital of the Federal University of Santa Catarina (Florianópolis, Brazil), Hospital Divino Espírito Santo (HDES), (Ponta Delgada, São Miguel, Portugal), and a private Laboratory of Pathology in Araçatuba, Brazil. All cases were reviewed by three pathologists (FM, FS and LV) on haematoxylin and eosin-stained (H&E) sections.

TMA construction

Representative areas of the invasive breast carcinomas were carefully selected on the H&E-stained sections and marked on individual paraffin blocks. Two tissue cores (2 mm in diameter) were obtained from each specimen and precisely deposited into a recipient paraffin block using a TMA workstation (TMA builder 20010.02, Histopatholoy Ltd, Hungary). Forty seven TMA blocks were constructed, each one containing 24 tissue cores, arranged in a 4x6 sector. In each TMA

block, normal breast and testicular tissue were included as controls. After construction, 2 μm tissue sections were cut and adhered to glass slides (Polysine*TM*, Menzel-Glasser, Germany) for the immunohistochemical studies and a H&E-stained section from each TMA block was reviewed in order to confirm the presence of morphological representative areas of the original lesions.

Immunohistochemistry

All the immunohistochemical assays were performed with specific monoclonal antibodies. Details about primary antibodies, antigen retrieval and IHC detection systems are described in Table1. Except for EGFR, in which epitope retrieval was performed by proteolytic enzyme digestion for 20 minutes (pepsin A, 4 g/l; Sigma-Aldrich, USA) at 37°C, all epitope retrieval was heat-induced at 98ºC in a water-bath during 30 minutes, using a commercially available citrate buffer solution (Vector Laboratories, USA), 1:100, pH=6.0, or an ethylenediaminetetraacetic (EDTA) solution (Novocastra, UK), 1:10, pH=9.0, as antigen unmasking solutions. After the respective antigen retrieval and washes in a phosphate buffer solution (PBS), endogenous peroxidase activity was blocked with a 3% hydrogen peroxide solution (Panreac, Spain) in methanol (Sigma-Aldrich, USA) for 10 minutes. The slides were incubated in a blocking serum (LabVision, USA) for 15 min and then incubated with the respective primary monoclonal antibodies. Immunoassays were performed using the streptavidin-biotin-peroxidase technique (SABC), (LabVision Corporation, Fremont, CA, USA) or the HRP labeled polymer (DakoCytomation, USA) detection system, according to manufacturer's instructions. All reactions were revealed with diamino-benzidine (DAB) chromogen (DakoCytomation). Tissues were then counterstained with Mayer's haematoxylin, dehydrated and coverslipped using a permanent mounting solution (Mounting Medium, Richard Allan Scientific, USA). Positive and negative controls were included in every set of reactions for each antibody used. Normal breast ducts and lobules present in many of the selected areas were also used as internal controls, as well as the non-neoplastic breast tissue cores included in each array. The evaluation of immunohistochemistry results was performed by three pathologists as follows: ER, PR and p63 were considered positive whenever more than 10% of the neoplastic cells showed nuclear staining; similarly, the same cutoff was used for CK5, CK14 and Vimentin cytoplasmic staining, as well as for P-cadherin membrane staining. Membrane expression for HER2 and EGFR was evaluated according to the DakoCytomation HercepTest® scoring system (Reis-Filho et al., 2005). Breast carcinomas were considered HER2-overexpressing whenever the immunohistochemical reaction was classified as 3+ or when gene amplification was confirmed by Chromogenic *In Situ* hybridization (CISH) in the 2+ cases, as described in other works (Ricardo et al., 2007). For EGFR, the cases were considered positive whenever the immunostaining was 2+ or 3+.

Hormone receptor (ER and PR) positive tumors were considered luminal A and B whether or not they overexpressed HER2, respectively (Sotiriou et al., 2003; Matos et al., 2005; Paredes et al., 2007b; Spitale et al., 2008; Tamimi et al., 2008). Cases lacking ER/PR with overexpression of HER2 were classified as HER2 overexpressing tumors. ER-/PR-/HER2- cases with immunoreactivity for EGFR and/or CK5 were considered BLBC according to the gold standard Nielsen's criterion and cases without expression of the five biomarkers were considered unclassified. When the immunoreactivity for the additional basal markers, namely P-cadherin, CK14 and Vimentin are used, the positive cases for at least one of these markers were considered as BLBC (P-cad and/or CK14 and/or Vim). Since for some markers the immunohistochemical result was not interpretable, the statistical analyses were performed using only 387 breast tumors cases which were classified for all the biomarkers tested.

Table 1. Conditions of the immunohistochemical reactions performed in this study.

	Primary antibodies				Antigen retrieval buffer	Detection method
Antigen	Clone	Origin	Incubation time (min)	Dilution		
ER	SP1	Neomarkers, USA	30	1:150	Citrate	SABC*
PR	SP2	Neomarkers, USA	30	1:300	Citrate	HRP-Polymer **
HER2	SP3	Neomarkers, USA	30	1:80	Citrate	SABC*
CK5	XM26	Neomarkers, USA	60	1:50	Tris-EDTA	SABC*
EGFR	31G7	Zymed	60	1:100	Pepsin	HRP-Polymer **
P-cadherin	56	BD Transduction	60	1:50	Tris-EDTA	HRP-Polymer **
CK14	LL002	Novocastra, UK	60	1:400	Tris-EDTA	HRP-Polymer **
Vimentin	V9	Dako, USA	30	1:150	Citrate	SABC*
p63	4A4	Neomarkers, USA	60	1:150	Citrate	SABC*

* SABC: streptavidin-avidin-biotin-complex; **: HRP-Polymer (horseradish peroxidase - polymer).

Statistical analysis

Statistical analysis was performed by SPSS statistics 17.0 (SPSS Inc., Chicago, IL, USA) software program. χ^2 contingency test was used to determine associations between groups and the results were considered statistically significant if the p value was lower than 0.05. In order to determine which were the most sensitive and specific biomarkers to identify BLBC, the sensitivity and the specificity of the antibodies used were calculated. Sensitivity measurement was defined by the quotient between the true positive (TrueP) cases and the sum of the true positive and the false negative (FalseN) cases [sensitivity = TrueP/(TrueP+FalseN)]. Specificity was measured in a similar way, by the quotient between the true negative (TrueN) cases with the sum of the true negatives and the false positives (FalseP) [specificity = TrueN/(TrueN+FalseP)]. PPV (Positive Predictive Value) and NPV (Negative Predictive Value) were calculated as follows: PPV = TrueP/(TrueP+FalseP) and PNV = TrueN/(TrueN+FalseN). As described before, ER/PR/HER2 negative tumors that express CK5/6 and/or EGFR were considered BLBC. Consequently, TrueP and TrueN cases were the BLBC tumors that were positive or negative, respectively, to the marker or pair of markers in analysis. Inversely, FalseP and FalseN were non BLBC positive or negative to the basal markers in study.

Follow-up information was available for 282 of the 387 cases and a maximum cutoff of 77 months was considered. Survival curves were estimated by the Kaplan-Meier method using log-rank test to assess significant differences for overall survival.

Results

In this series of 387 breast carcinomas, 223/387 (57.6%) and 144/387 (37.2%) cases were ER and PR positive, respectively, and 65/387(16.8%) overexpressed HER2. Using the ER/PR/HER2- (TN) criterion, this series comprises 109 (28.2%) triple negative and 278 (71.8%) non-Triple Negative tumors. Considering the molecular subtypes of breast cancer, 213 (55%) cases were luminal A, 13 (3.4%) luminal B and 52 (13.4%) HER2-overexpressing tumors. According to Nielsen's criterion, 37 (9.6%) cases presented a basal-like phenotype and 72 (18.6%) were considered "unclassified" by this criterion. We analyzed the associations between CK5, EGFR, P-cadherin, CK14, p63 and Vimentin and the BLBC versus non BLBC (Table 2). As expected, the markers were significantly associated with the basal phenotype ($p<0.0001$), with the exception for p63 ($p=0.5403$). Fig. 1 shows the immunohistochemical staining for CK5, EGFR, P-cadherin, Vimentin and CK14 in BLBC.

Afterwards, the sensitivity, specificity, PPV and NPV of each biomarker for the identification of BLBC were calculated (Table 3), except for p63 which was not even related with basal phenotype. CK5 was the most sensitive biomarker (91.9%), followed by P-cadherin (67.6%). CK14 and EGFR were the most specific markers, presenting 98.6% and 97.1% of specificity, respectively, and vimentin was also shown to be very specific (86.9%).

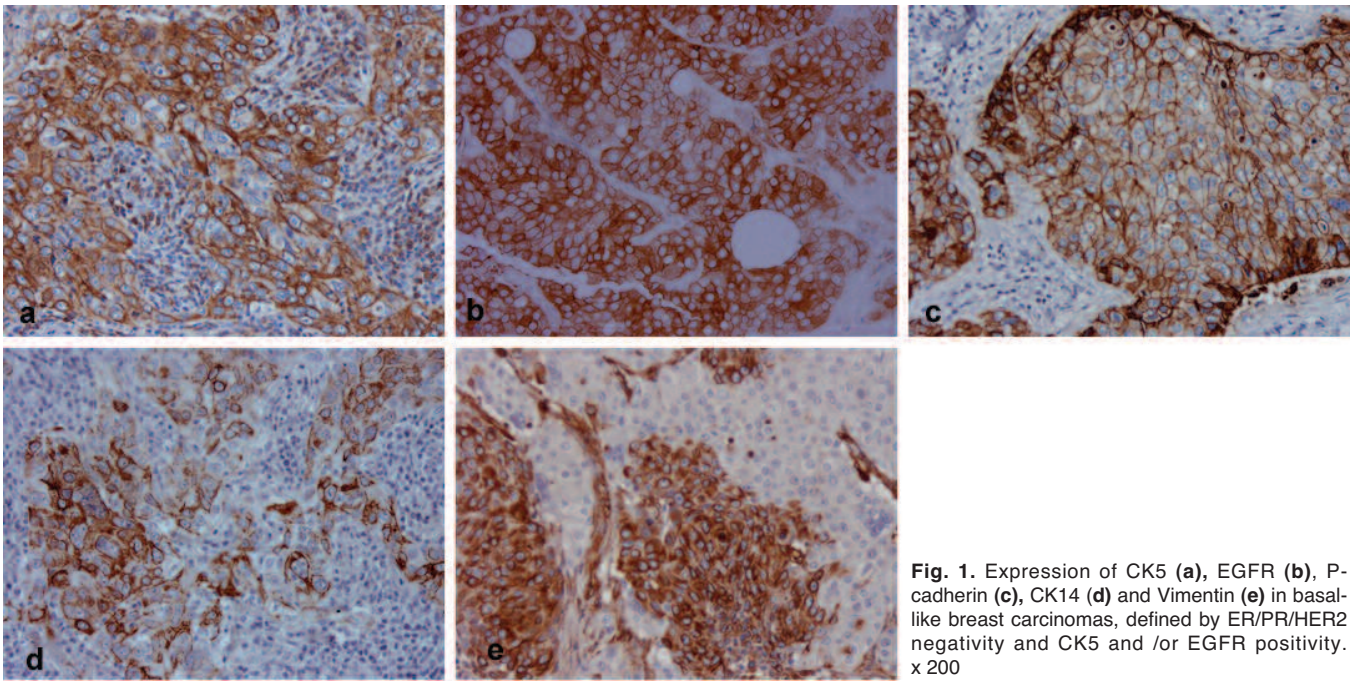

Fig. 1. Expression of CK5 **(a),** EGFR **(b)**, P-cadherin **(c),** CK14 **(d)** and Vimentin **(e)** in basal-like breast carcinomas, defined by ER/PR/HER2 negativity and CK5 and /or EGFR positivity. x 200

In order to find the best combination of basal markers with the ability to identify BLBC, we evaluated the most sensitive and the most specific markers in pairs (CK5, P-cadherin with CK14, EGFR or Vimentin). Since P-cadherin presented good sensitivity and specificity values, we also evaluated its association with CK5 (Table 4). The statistical associations considered cases that were positive for both markers (+/+), positive for at least one marker (+/- or -/+) or negative for both (-/-). Table 5 shows the percentages of sensitivity, specificity, PPV and NPV for the several pairs of markers. In these analyses, we considered as true positive the cases that were +/+ and positive for at least one of the markers in the subgroup of BLBC previously distinguished by Nielsen's criterion, and as false positive the cases that

Table 2. Association between the expression of CK5, EGFR, P-cadherin, CK14, p63 and vimentin with basal-like and non basal-like breast carcinomas.

	n	Basal n (%)	Non basal n(%)	P
	387	37(9.6%)	350(90.4%)	
CK5				<0.0001
+	89	34(91.9%)	55(15.7%)	
-	298	3(8.1%)	295(84.3%)	
EGFR				<0.0001
+	21	11(29.7%)	10(2.9%)	
-	366	26(70.3%)	340(97.1%)	
P-cadherin				<0.0001
+	123	25(67.6%)	98(28%)	
-	264	12(32.4%)	252(72%)	
CK14				<0.0001
+	17	12(32.4%)	5(1.4%)	
-	370	25(67.6%)	345(98.6%)	
p63				0.5403
+	14	2(5.4%)	12(3.4%)	
-	373	35(94.6%)	338(96.6%)	
Vimentin				<0.0001
+	63	17(45.9%)	46(13.1%)	
-	324	20(54.1%)	304(86.9%)	

Table 3. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the IHC method for the basal-markers studied to discriminate a basal-like carcinoma.

	Sensitivity (%)	Specificity (%)	PPV (%)	PNV (%)
CK5	91.9	84.3	38.2	99.0
EGFR	29.7	97.1	52.4	92.9
P-cadherin	67.6	72.0	20.3	95.5
CK14	32.4	98.6	70.6	93.2
Vimentin	45.9	86.9	27.0	93.8

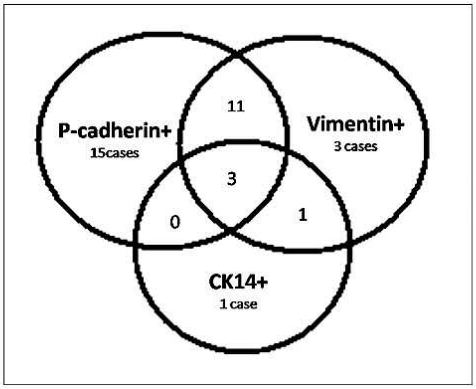

Fig. 2. Distribution of P-cadherin, vimentin and CK14 expression in triple negative tumors that were negative for CK5 and EGFR.

Table 4. Association between the expression of pairs of basal markers with basal-like and non basal-like breast carcinomas.

	n	Basal n (%)	Non basal n(%)	p
CK5/EGFR				<0.0001
+/+	11	8(21.6%)	3(0.8%)	
At least one +	88	29(78.4%)	59(16.9%)	
-/-	288	0(0%)	288(82.3%)	
CK5/CK14				<0.0001
+/+	11	11(29.7%)	0(0%)	
At least one +	83	23(62.2%)	60(17.1%)	
-/-	293	3(8.1%)	290(82.9%)	
CK5/Vim				<0.0001
+/+	24	16(43.2%)	8(2.3%)	
At least one +	104	19(51.4%)	85(24.3%)	
-/-	259	2(5.4%)	257(73.4%)	
P-cadherin/EGFR				<0.0001
+/+	13	8(21.6%)	5(1.4%)	
At least one +	118	20(54.1%)	98(28%)	
-/-	256	9(24.3%)	247(70.6%)	
P-cadherin/CK14				<0.0001
+/+	12	9(24.3%)	3(0.9%)	
At least one +	116	19(51.4%)	97(27.7%)	
-/-	259	9(24.3%)	250(71.4%)	
P-cadherin/Vim				<0.0001
+/+	41	11(29.7%)	30(8.6%)	
At least one +	104	20(54.1%)	84(24%)	
-/-	242	6(16.2%)	236(67.4%)	
P-cadherin/CK5				<0.0001
+/+	38	23(62.2%)	15(4.3%)	
At least one +	136	13(35.1%)	123(35.1%)	
-/-	213	1(2.7%)	212(60.6%)	

Table 5. Sensitivity, specificity, PPV and NPV of the IHC method for the pairs of basal-markers antibodies studied to discriminate a basal-like carcinoma.

	Sensitivity (%)	Specificity (%)	PPV (%)	PNV (%)
CK5/EGFR	100	82.3	11.4	100
CK5/CK14	91.9	82.9	10.5	99
CK5/Vim	94.6	73.4	12.0	99.2
P-cadherin/EGFR	75.7	70.6	10.2	96.5
P-cadherin/CK14	75.7	71.4	10.1	96.5
P-cadherin/Vim	83.8	67.4	11.6	97.5
P-cadherin/CK5	97.3	60.6	14.5	99.5

were positive for the two markers and the ones expressing at least one marker in non basal-like tumors. True negative and false negative were the -/- cases in non basal-like and in BLBC, respectively. All the associations were statistically significant ($p<0.0001$). The pair CK5/EGFR presented, as expected, the highest values of sensitivity and specificity, 100% and 82.3%, respectively. However, concerning sensitivity, the pairs CK5/CK14, P-cadherin/CK5 and CK5/Vimentin showed similar values to the "gold standard" CK5/EGFR pair, with 91.9%, 97.3% and 94.6% of sensitivity, respectively. The specificity of CK5/CK14 combination (82.9%) was approximately equal to the one presented by CK5/EGFR (82.3%).

In the BLBC group, when analyzing the number of cases that were +/+ and positive for at least one of the markers of the pair, against the -/- cases (Table 6), it is possible to observe that only one basal-like breast carcinoma was negative for both markers in P-cadherin/CK5 pair. The CK5/Vimentin pair missed the expression in 2 cases, while CK5/CK14 did not stain three BLBC. All the other pairs were positive in BLBC for the two markers, or for at least one of them, in at least 75.7% of breast carcinomas with basal phenotype.

More importantly, given the sensitivity of P-cadherin and the specificity of CK14 and Vimentin, we also analyzed their expression among the TN/CK5 and EGFR

Table 6. Analyzes of the distribution of expression of the pairs of markers in BLBC.

		Basal n (%)
CK5/EGFR	+/+ and at least one + -/-	37(100%) 0(0%)
CK5/CK14	+/+ and at least one + -/-	34(91.9%) 3(9.1%)
CK5/Vim	+/+ and at least one + -/-	35(94.6%) 2(5.4%)
P-cadherin/EGFR	+/+ and at least one + -/-	28(75.7%) 9(24.3%)
P-cadherin/CK14	+/+ and at least one + -/-	28(75.7%) 9(24.3%)
P-cadherin/Vim	+/+ and at least one + -/-	31(83.8%) 6(16.2%)
P-cadherin/CK5	+/+ and at least one + -/-	36(97.3%) 1(2.7%)

Table 7. Expression of P-cadherin, vimentin and CK14 in the 72 TN tumors also negative for CK5 and EGFR.

		TN/CK5 and EGFR- n=72
P-cadherin	+ -	29(40.3%) 43(59.7%)
Vimentin	+ -	18(25%) 54(75%)
CK14	+ -	5(6.9%) 67(93.1%)

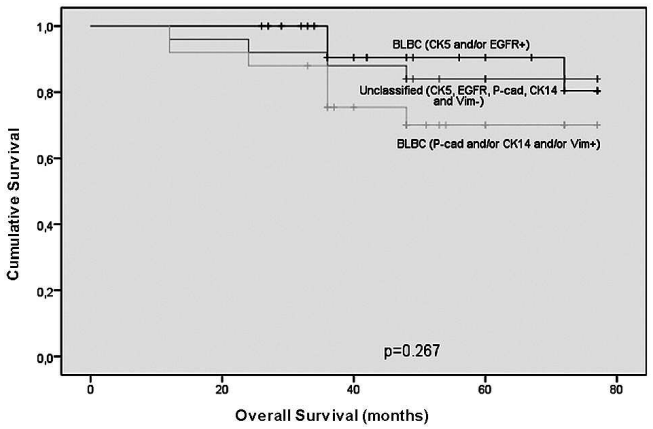

Fig. 3. Kaplan-Meier survival curves for overall survival (OS) of triple negative breast carcinoma patient's cohort, with a 77 months cut-off. BLBC defined by TN/CK5 and/or EGFR+ [BLBC (CK5 and/or EGFR+)], BLBC defined as ER/PR/HER2-, CK5/EGFR- and immunoreactivity for P-cadherin and/or CK14 and/or Vimentin [BLBC (P-cad and/or CK14 and/or Vim)] and tumors that were negative for all the basal markers in study were analyzed, p=0.267 (not statistically significant).

Table 8. Distribution of histological grade among triple negative breast carcinomas of the studied series.

	Histological grade		
Triple negative tumors (n=103*)	I	II	III
BLBC (CK5 and/or EGFR+) (n=34)	3 (9%)	12 (35%)	19 (56%)
BLBC (P-cadherin and/or CK14 and/or Vimentin+) (n=32)	2 (6%)	15 (47%)	15 (47%)
Unclassified (TN,CK5, EGFR, P-cad, CK14 and Vim-) (n=37)	17 (46%)	15 (40%)	5 (14%)

BLBC (CK5 and/or EGFR+) are the TN tumors that were positive for CK5 and/or EGFR and BLBC (P-cadherin and/or CK14 and/or Vimentin+) are the TN/CK5 and EGFR- tumors immunoreactive for one of the additional markers in study: P-cadherin, CK14 and vimentin. *: Histological grade of some cases could not be assessed because the patients were submitted to preoperative chemotherapy.

negative tumors ("unclassified" by Nielsen's criterion). In 38/72 (52.8%) cases, none of the biomarkers were expressed; however, in the other 34/72 cases (47.2%), there was the expression of, at least, one of the biomarkers. P-cadherin was present in 29 (40.3%), Vimentin in 18 (25%) and CK14 in 5 (6.9%) of these tumors (Table 7). In a more detailed analysis, 15 cases were positive only for P-cadherin, while only one and three cases were positive for CK14 and for Vimentin alone, respectively (Fig. 2).

Interestingly, if we consider as BLBC these TN/CK5 and EGFR- "unclassified" cases that presented immunoreactivity for P-cadherin, CK14 and/or Vimentin [BLBC (Pcad and/or CK14 and/or Vimentin+)], this series presents 71/387 (18%) of BLBC. BLBC defined by TN/CK5 and/or EGFR+ and BLBC defined as ER/PR/HER2-, CK5/EGFR- and immunoreactivity for P-cadherin and/or CK14 and/or Vimentin were analyzed separately. These two differently defined BLBC presented a similar percentage of high histological grade tumors [56% and 47% in BLBC (CK5 and/or EGFR+) and in BLBC (Pcad and/or CK14 and/or Vimentin+), respectively], (Table 8). The overall survival was similar for the two groups as we can see in Figure 3.

Discussion

The need for a more precise diagnosis of breast cancer that converges with the clinical outcome and the choice of the most appropriate therapy has motivated studies in different areas of breast cancer research. The cDNA microarray technology is a "gold standard" method for the recognition of the basal phenotype, but from a practical point of view, we need to translate these results to an accessible method. It is undeniable that the BLBC immunohistochemistry definition requires cDNA microarray validation, since these tumors were first identified by this technique (Perou et al., 2000; Livasy et al., 2006). However, from the pathologists and oncologists point of view, the lack of molecular targets for therapy in this subgroup of patients indicates the urgent need for an easier and less expensive way to identify BLBC patients. Based on this, there is an attempt to establish an immunohistochemical surrogate panel, easily applied on FFPE samples, which identifies a pool of breast cancer patients who may require more aggressive systemic therapy and that would be the most appropriate subjects for clinical trials, specifically targeting this molecular subgroup of breast cancer. However, there is still no consensual definition about the ideal IHC panel of biomarkers to distinguish the basal phenotype. In fact, many different panels have been used, in which CK5, EGFR, P-cadherin, CK14 and Vimentin are included. Due to this diversity of criteria, a wide range of percentages of BLBC are described in the several studied series (van de Rijn et al., 2002; Foulkes et al., 2004; Jones et al., 2004; Abd El-Rehim et al., 2005; Arnes et al., 2005; Collett et al., 2005; Kusinska et al., 2005; Laakso et al., 2005; Potemski et al., 2005; Banerjee et al., 2006; Fulford et al., 2006, 2007; Kim et al., 2006; Rakha et al., 2006a,b, 2007a,b,c; Rodriguez-Pinilla et al., 2006, 2007; Siziopikou and Cobleigh, 2007). Nielsen et al. (2004) demonstrated that CK5 and EGFR could reliably discriminate BLBC that were identified by GEP, considering these two basal markers the "gold standard" immunohistochemical panel of antibodies to the BLBC identification, together with ER and HER2 lack of expression. Recently, Cheang et al. (2008) compared two BLBC immuno-panels and concluded that the ER-/PR-/HER2- and expression of CK5 and/or EGFR provides the more accurate definition of BLBC and can better predict breast cancer patient's survival.

However, we cannot assure which are the best antibodies to be included in a daily practice panel for the recognition of the basal phenotype in breast carcinomas: should we look for the most sensitive or the most specific ones? None of these markers are actually pathognomonic of a basal phenotype, since they are variably expressed in the other subgroups of breast carcinomas, which support the search for "ideal" biomarkers to be used in the anatomic pathology workup and with clinical relevance.

We demonstrate herein that P-cadherin, Vimentin or CK14 may possibly be useful biomarkers to include in IHC panels for distinguishing BLBC. P-cadherin reveals consistent values of sensitivity and specificity, while Vimentin and CK14 presented high specificity values. The three markers were able to reliably recognize the basal phenotype, especially when associated to CK5.

The presence of P-cadherin, an adhesion molecule expressed in myoepithelial cells of the normal mammary gland, was already described in invasive and in *in situ* breast carcinomas with worst prognosis, namely in those with high histological grade and basal phenotype (Peralta Soler et al., 1999; Gamallo et al., 2001; Kovacs and Walker, 2003; Paredes et al., 2005, 2007b). The role of P-cadherin in breast carcinogenesis has been one of the main fields of our research group's interest and we have observed that this molecule presents an inverse correlation with HR (Peralta Soler et al., 1999; Gamallo et al., 2001; Kovacs and Walker, 2003; Paredes et al., 2005) and a direct correlation with EGFR (Kovacs and Walker, 2003), HER2 and high proliferation rates, strengthening the value of P-cadherin as a poor prognostic indicator in breast cancer (Palacios et al., 1995; Peralta Soler et al., 1999; Gamallo et al., 2001; Paredes et al., 2005). The expression of P-cadherin in neoplastic cells has already been related to a histogenetic origin in cap cells or to the acquisition of a stem cell-like phenotype, suggesting that P-cadherin-expressing tumors could be associated to a stem cell origin (Peralta Soler et al., 1999, Gamallo et al., 2001, Paredes et al., 2007). Recently, it has been suggested that basal-like breast carcinomas may be genuine stem/early progenitor cell tumors of the mammary gland, relating their origin to a more undifferentiated type of precursor cells (Honeth et al., 2008). Also, Rakha et al. (2009)

demonstrated more evidence of the features of dual-lineage differentiation/stem cell phenotype of BLBC by showing a higher frequency of CK19 expression in this type of tumor.

CK14 does not show a differential presence in breast carcinomas with basal phenotype identified by cDNA microarray technology, but this cytokeratin is frequently associated with poor prognosis (Jones et al., 2004) and with the morphological features observed in BLBC (Tsuda et al., 2000). For this reason, CK14 has been included in the immunopanel used to identify BLBC by several other authors (Laakso et al., 2005, 2006; Rakha et al., 2006a,b; Reis-Filho et al., 2006).

Vimentin is an intermediate filament protein whose expression in normal mammary gland is also restricted to myoepithelial/ basal layer. Its expression has been associated with high histological grade, lack of ER, p53 mutations, high proliferation rates (Raymond and Leong, 1989; Domagala et al., 1990a,b; Koutselini et al., 1995; Santini et al., 1996; Thomas et al., 1999) and expression of CK5/6 and EGFR (Korsching et al., 2005; Reis-Filho, 2005). Vimentin-expressing carcinomas have been observed in association with sporadic and familial BLBC and with a specific pattern of metastasis similar to BLBC (Rodriguez-Pinilla et al., 2007). Like P-cadherin, Vimentin was also described to be differentially expressed by BLBC identified by GEP, being proposed to integrate the panel of antibodies for the identification of BLBC (Livasy et al., 2006).

Our results show that P-cadherin, CK14 and Vimentin, together with CK5, can identify almost all BLBC that were classified as such using the most widely accepted IHC panel to classify BLBC: ER/PR/HER2- and CK5 and/or EGFR+.

Triple negative phenotype by IHC is one of the characteristic features of BLBC and several authors claim that basal tumors are almost all TN tumors (Diaz et al., 2007; Kreike et al., 2007). Kreike et al. (2007), in a series of 97 TN cases, observed that 90% of these tumors have a basal phenotype by cDNA microarray analysis. However, the lack of expression of ER, PR and HER2 as the sole criterion to identify these tumors is risky (Rakha et al., 2008) because there are technique limitations when dealing with FFPE tissue samples, which reinforces the need for a more suitable panel.

There is a significant overlapping of features shared by triple negative and BLBC in what concerns, for example, the prevalence of these types of cancer in younger patients, in African-American women (Morris et al., 2007), their presentation as interval cancers, a similar pattern of recurrence (Dent et al., 2007; Tischkowitz et al., 2007), the more aggressive behavior comparing with other types of breast cancer (Reis-Filho and Tutt, 2008) and the biological and clinical similarity between sporadic TN and BLBC with breast carcinomas arising from BRCA1 mutation carriers (Reis-Filho and Tutt, 2008). However, several studies claim that this overlap is not complete (Bertucci et al., 2008, Rakha and Ellis, 2009). It is known that TN carcinomas with basal phenotype have a significant shorter disease-free survival than TN without expression of basal markers (Rakha et al., 2007a; Tischkowitz et al., 2007) and that germline BRCA1 mutation carriers are more probably found in TN tumors expressing CK5/6 and /or EGFR than in TN with no expression of these basal markers (Turner et al., 2007; Rakha et al., 2009). It has also been observed in GEP that triple negative group is composed by other subgroups of tumors with different outcomes, namely the normal breast-like tumors (Perou et al., 2000; Sorlie et al., 2001, 2003; Sotiriou et al., 2003; Fan et al., 2006; Hu et al., 2006; Hennessy et al., 2009) and a recently described subgroup of claudin-low tumors (Herschkowitz et al., 2007; Hennessy et al., 2009). The existence of TN tumors that do not react immunohistochemically with any of the basal markers routinely used has been described, and variably designated as non basal triple negative, unclassified, undetermined, null phenotype (Liu et al., 2008) or TN3BKE- (Triple Negative 3 Basal Keratins and EGFR-) (Rakha et al., 2009). It seems extremely important to distinguish BLBC from the whole triple negative group, reducing the TN heterogeneity, since their biological behavior appears to be different. The lightening of this heterogeneity would enable patients to benefit from their differential recognition (Rakha et al., 2007a, 2008, 2009; Liu et al., 2008; Reis-Filho and Tutt, 2008; Tan et al., 2008; Rakha and Ellis, 2009). This distinction is also important because TN tumors defined by IHC tend to be clinically considered as BLBC and selected for clinical trials (Bertucci et al., 2008), probably misleading the effect of the drugs in the clinical trials.

It is interesting to emphasize that among the analyzed TN/CK5 and EGFR- tumors that were also negative for P-cadherin, CK14 and Vimentin, approximately 50% of these cases presented low histological grade (Table 8). P-cadherin was expressed alone in a higher number (15 cases) of TN/CK5 and EGFR negative tumors, compared with CK14 (1 case) and Vimentin (3 cases). When P-cadherin, CK14 and Vimentin expression are considered along with CK5 and EGFR for the BLBC identification, 34 cases are added to the 37 already identified BLBC (CK5 and/or EGFR+) and the percentage of basal-like tumors in the pool of TN cases of our series rounds the 65% (71/109). This rate is similar to the one identified by Bertucci (Bertucci et al., 2008), where 70% of IHQ TN tumors presented a basal phenotype by GEP. It is worth noticing that using P-cadherin, CK14 and Vimentin to recruit BLBC from the pool of tumors that could not be classified using only CK5 and EGFR as basal makers, these newly identified BLBC are clinically similar to basal-like tumors identified by Nielsen's criterion, since the majority of the cases presented high histological grade and there are no significant differences in what concerns overall survival of the patients.

Although CK5 and EGFR have been consistently used to recognize BLBC, P-cadherin, CK14 and

Vimentin could also be recruited for an immunohistochemical recognition of BLBC (Paredes et al., 2002, 2007a,b; Matos et al., 2005; Livasy et al., 2006; Rodriguez-Pinilla et al., 2007). Our results showed that these three markers can reliably identify the basal phenotype, especially when associated to CK5, and can be alternative options in this setting. We also demonstrate that P-cadherin, due to its high sensitivity, can recognize possible BLBC among the IHC TN tumors, probably identifying patients with poor prognosis that can benefit from this differential recognition. Pathologists have faced continuous changes in the diagnostic approach of breast cancer and, regarding its classification, it is still controversial whether or not the histological classification should be replaced by the "molecular" taxonomy. Therefore, it is essential to move towards a standardized methodology to establish an IHC panel of biomarkers to the most appropriate recognition of basal-like breast carcinomas.

Conflict of interest. The authors declare that they have no conflict of interest.

Acknowledgements. This work was partially supported by research grants from Bárbara Sousa (collaboration project at IPATIMUP); Joana Paredes: Ciência 2007 – Portuguese Science and Technology Foundation (FCT); Nair Lopes: FCT-SFRH/BD/39208/2007; Diana Martins: FCT- SFRH/BD/66152/2009; André Albergaria: FCT-SFRH/BD/15316/2005; Silvia Carvalho: FCT-SFRH/BD/21551/2005 and José Costa: FCT-SFRH/BPD/20370/2004.

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Accepted February 5, 2010

PUBLICATIONS

Paper 6

Histopathology 2010, **56,** 860–867. DOI: 10.1111/j.1365-2559.2010.03560.x

Monocarboxylate transporter 1 is up-regulated in basal-like breast carcinoma

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Date of submission 10 March 2009
Accepted for publication 1 September 2009

Pinheiro C, Albergaria A, Paredes J, Sousa B, Dufloth R, Vieira D, Schmitt F & Baltazar F (2010) *Histopathology* **56,** 860–867

Monocarboxylate transporter 1 is up-regulated in basal-like breast carcinoma

Aims: Monocarboxylate transporters (MCTs) have been considered promising targets for cancer therapy, since they facilitate lactate efflux in glycolytic tumours. However, their role in solid tumours is still poorly understood. Thus, the present work aimed to contribute to understanding the involvement of MCT1 and MCT4 in breast cancer progression as well as MCT regulation by CD147.

Methods and results: The expression of the membrane transporters MCT1 and MCT4 was analysed in a series of breast carcinomas (249 cases) and their clinicopathological significance investigated. Additionally, we analysed the significance of CD147 co-expression, as an important regulator of MCT expression and activity. MCT1 was significantly increased in breast carcinomas when compared with normal breast tissue and, importantly, both MCT1 and CD147 were associated with poor prognostic variables such as basal-like subtype and high grade tumours.

Conclusions: These results provide evidence for a prognostic value of MCT1 in breast carcinoma and support the exploitation of the complex MCT1/CD147 as a promising target for cancer therapy, especially in basal-like breast carcinoma.

Keywords: breast carcinoma, CD147, immunohistochemistry, monocarboxylate transporter

Abbreviations: CK, cytokeratin; DAB, 3,3′-diaminobenzidine; DCIS, ductal carcinoma *in situ;* EGFR, epidermal growth factor receptor; ER, oestrogen receptor; MCTs, monocarboxylate transporters; PR, progesterone receptor; TMA, tissue microarray

Introduction

Despite advances in cancer therapy, breast cancer is still the leading cause of cancer-related death in women worldwide.[1] Breast cancer can be classified according to gene expression profiles into four main groups: basal-like, luminal (A and B), HER2+ and normal-like breast carcinomas,[2–7] which have important prognostic implications: basal-like and HER2+ tumours have more aggressive clinical behaviour when compared with luminal and normal-like breast carcinomas.[2–4,6,7] Basal-like breast carcinomas, in contrast to the other groups, do not have a specific molecular therapy, necessitating the search for new molecular targets in this aggressive group of tumours.[8,9]

The extracellular pH of solid tumours is generally low, whereas cytosolic pH is normal or higher than that of normal tissues.[10,11] In breast cancer cells, this lower extracellular pH is explained by, *inter alia*, two complementary mechanisms: increased lactate production through glycolysis and proton-pump activity in the plasma membrane. The former, which is quantitatively more important, is consistent with the

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known high glycolytic rates of cancer cells, especially under anaerobic conditions, which results in production of large amounts of lactate.[12] Acidification of the extracellular microenvironment has several implications in tumour progression, e.g. up-regulation of various angiogenic molecules including vascular endothelial growth factor,[13,14] which support tumour growth, invasion and metastasis, and up-regulation of proteases implicated in cancer cell invasiveness, through digestion of the extracellular matrix.[14]

One of the most important mechanisms involved in intracellular pH regulation is the co-transport of lactate and a proton, which is mediated by a family of membrane proteins known as monocarboxylate transporters (MCTs).[15] These proteins are also responsible for the transport of pyruvate and ketone bodies, being critical for metabolic communication between cells.[16] Taking into consideration the high glycolytic rates of cancer cells, it is to be expected that MCTs would be up-regulated, to allow maintenance of glycolysis rates and prevent apoptosis by intracellular acidosis. Indeed, there is evidence for the up-regulation of MCTs in several tumours.[17–24] However, only a few studies have evaluated the clinicopathological significance of MCT overexpression.[23–25] We have recently described MCT1 and MCT4 overexpression in colorectal carcinomas, as well as an association between plasma membrane expression of MCT1 and vascular invasion.[24] Furthermore, we observed an increase in MCT1 and MCT4 during progression to invasive cervical carcinoma and an association between both MCT1 and MCT4 expression and high-risk human papillomavirus infection.[23] Moreover, we observed preferential expression of MCT4 in intestinal-type gastric carcinoma, although with a decrease in MCT4 expression from normal to malignant gastric mucosa.[25]

To the best of our knowledge, MCT analysis in breast cancer samples has been performed in only one study, where silencing of MCT1 by methylation of the 5′ region of the gene was suggested, in four of 19 breast cancer samples.[26] However, this result is far from clarifying the role of MCTs in breast cancer development and/or progression.

It was recently demonstrated that CD147, a known key regulator of oncogenesis,[27–29] is an ancillary protein required for cell surface expression and activity of MCT1 and MCT4.[30–32] On the other hand, silencing studies have shown that maturation and cell surface expression of CD147 depends on both MCT1 and MCT4 expression.[33,34] Recently, and supporting this evidence, we described a close association between both MCT1 and MCT4 and CD147 in cervical cancer.[35]

In the present work, we sought to assess the involvement of MCT1 and MCT4 in breast cancer progression by analysing the expression of these membrane transporters in a series of breast carcinomas, and to investigate its clinicopathological significance. We also aimed to contribute to the understanding of MCT regulation by CD147 in tumours, by analysing the significance of MCT and CD147 co-expression in breast carcinoma.

Materials and methods

CASE SELECTION

Case selection was based on availability of follow-up information and amount of material, ensuring adequate numbers for statistical analysis. Thus, a series of 249 formalin-fixed paraffin-embedded primary breast carcinomas was retrieved from the files of the Department of Pathology, Hospital do Divino Espírito Santo (Azores, Portugal) and from the Federal University of Santa Catarina (Florianópolis-SC, Brazil). Samples were obtained from patients aged 30–89 years. Haematoxylin and eosin-stained sections of all cases were reviewed by three pathologists (R.D., D.V. and F.S.) and the diagnoses were confirmed as follows: 208 invasive ductal carcinomas, seven invasive lobular carcinomas, three mixed lobular-ductal carcinomas, three tubular, eight medullary and 20 invasive breast carcinomas of other special histological types. Representative areas of tumour samples, as well as 53 samples of normal breast tissue, mostly from the same breast with tumour, were carefully selected and organized into tissue microarrays (TMAs), with cores of 2 mm diameter. Each case was represented in the TMA by at least two cores. In 45 cases, areas of ductal carcinoma *in situ* (DCIS) were present and were also analysed. Relevant clinicopathological data from these tumours included tumour size, molecular classification, histological grade and lymph node metastasis. The distribution of prognostic factors with a high number of cases with lymph node metastasis and oestrogen receptor (ER)-negative status, reflects a large number of cancer patients with advanced disease.

The molecular classification was carried out based on immunohistochemical results for ER, progesterone receptor (PR), HER2, epidermal growth factor receptor, cytokeratin (CK) 5, CK14, vimentin and Ki67. Tumours positive for ER and/or PR were classified as luminal. Cases positive for ER/PR and for HER2 and/or high Ki67 index were subclassified as luminal B. Cases classified as HER2 overexpressing were characterized by HER2 overexpression and negativity for ER/PR, and cases defined as 'basal-like' were negative

for ER/PR and HER2 and positive for at least one of the 'basal markers' tested.

Follow-up information was available for 218 cases, ranging from a minimum of two to a maximum of 129 months (median 32 months). Disease-free survival interval data were evaluated and defined as the time from the date of surgery to the date of breast cancer-derived relapse/metastasis. Due to the short follow-up of the studied series and consequent limited number of death events, overall survival was not analysed.

This study was conducted under the relevant national law regulating the usage of biological specimens from tumour banks, where the samples are exclusively available for research purposes in the case of retrospective studies.

IMMUNOHISTOCHEMISTRY

MCT detection

Immunohistochemistry was performed according to the avidin–biotin–peroxidase complex principle [R.T.U. VECTASTAIN Elite ABC Kit (Universal); Vector Laboratories, Burlingame, CA, USA], with the primary antibodies for MCT1 (AB3538P; Chemicon International, Temecula, CA, USA) and MCT4 (AB3316P; Chemicon International), diluted 1:200 for MCT1 and 1:100 for MCT4, as previously described.[23–25] Briefly, deparaffinized and rehydrated tissue sections were submitted to antigen retrieval by immersion in citrate buffer (0.01 M, pH 6.0) and heated to 98°C for 20 min, in a water bath. Tissues were then incubated with the primary antibody overnight at room temperature and immunoreactivity was visualized with 3,3′-diaminobenzidine (DAB+ Substrate System; Dako, Carpinteria, CA, USA). Colonic carcinoma was used as positive control for both MCT1 and MCT4.

CD147 detection

Immunohistochemistry was performed according to the avidin–biotin–peroxidase complex principle (Ultravision Detection System Anti-polyvalent, Horseradish peroxidase; Lab Vision Corp., Fremont, CA, USA), using a primary antibody raised against CD147 (18-7344; Zymed Laboratories Inc., South San Francisco, CA, USA) diluted 1:750, as previously described.[35] Briefly, deparaffinized and rehydrated tissue sections were submitted to antigen retrieval by immersion in ethylenediamine tetraaceticacid (pH 8.0) heated to 98°C for 15 min, in a water bath. Tissues were then incubated with the primary antibody, 2 h at room temperature, and immunoreactivity was visualized with DAB (DAB+ Substrate System; Dako). Cervical squamous carcinoma was used as positive control.

Immunohistochemical evaluation

Since plasma membrane location is essential for protein activity, immunoreactions for MCTs and CD147 were considered positive only when plasma membrane reactivity was present. Sections were scored semiquantitatively in relation to the positive control as previously described:[23–25,35] 0, 0% of immunoreactive cells; 1, <5% of immunoreactive cells; 2, 5–50% of immunoreactive cells; and 3, >50% of immunoreactive cells. Also, intensity of staining was scored as: 0, negative; 1, weak; 2, intermediate; and 3, strong. Final immunoreactivity score was defined as the sum of both parameters (extent and intensity), and grouped as negative (score 0 and 2) and positive (3–6). Evaluation was performed blindly by two independent observers and discordant results were discussed at a double-head microscope to reach the final score.

STATISTICAL ANALYSIS

Data were stored and analysed using Statview statistical software (SAS Institute Inc., Cary, NC, USA). All comparisons were examined for statistical significance using Pearson's chi-squared test or Fisher's exact test, as appropriate; the threshold for significance was set at $P < 0.05$. Disease-free survival curves were estimated by the method of Kaplan–Meier and data compared using the log rank test. A cut-off of 60 months (5 years) was considered, since in the first 5 years following primary therapy recurrence rates are expected to be highest, especially in series with high numbers of ER–cases like ours, where the hazard of recurrence is higher.[36] Cases lacking one or more of the clinicopathological variables were not included in the specific statistical analysis.

Results

A total of 249 breast carcinoma samples for MCT1, MCT4 and CD147 immunohistochemical expression were analysed. Due to technical problems (mainly tissue loss during immunohistochemistry), some cases are missing in the expression analysis.

MCT1 expression was mainly found at the plasma membrane whereas MCT4 was observed both in the cytoplasm and plasma membrane (Figure 1A,B). CD147 was mainly detected at the plasma membrane (Figure 1C), with some cases also showing cytoplasmic immunoreactivity. Table 1 summarizes the frequency of MCT and CD147 expression in non-neoplastic mammary epithelium and tumour samples. A significant increase in MCT1 expression was observed in tumours when compared with normal tissues

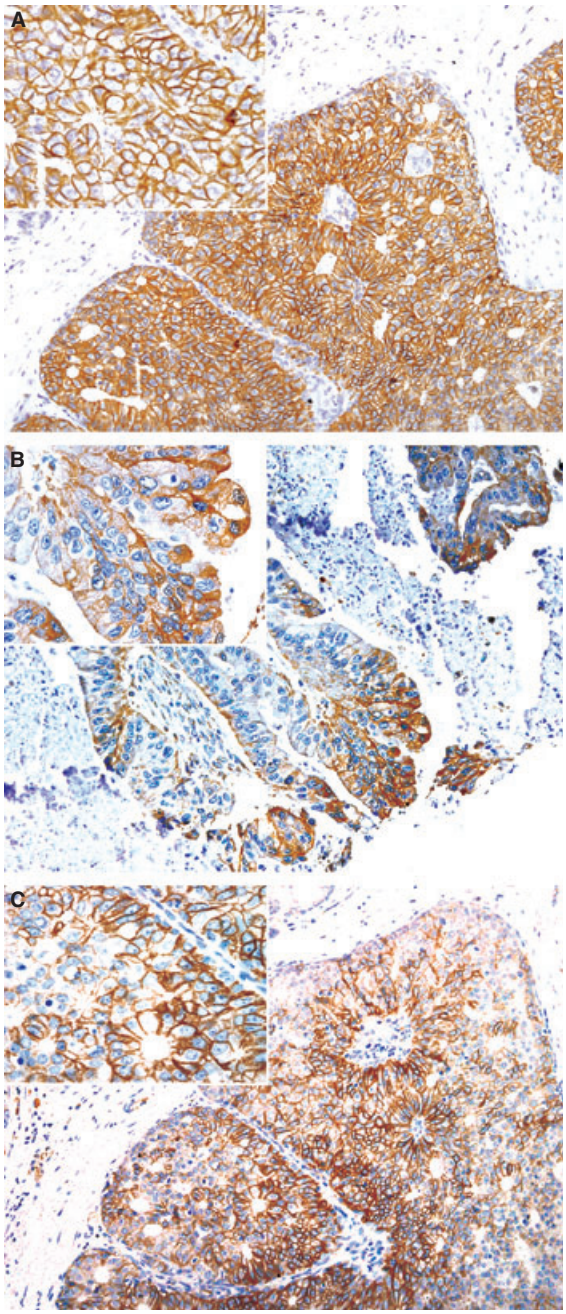

Figure 1. Immunohistochemical expression of monocarboxylate transporter (MCT) 1, MCT4 and CD147 in breast carcinoma samples. MCT1 expression is mainly found on the plasma membrane of tumour cells (**A**), whereas MCT4 reactivity is found both in the cytoplasm and plasma membrane (**B**). CD147 is mainly observed on the plasma membrane of tumour cells (**C**).

($P = 0.0138$), with 19.5% of tumours showing MCT1 expression, while no significant difference was observed for MCT4 and CD147.

Analysis of CD147 and MCT plasma membrane expression showed an association between CD147 and both MCT1 and MCT4 ($P < 0.0001$ and $P = 0.0083$, respectively, Table 2). DCIS, when present in tissue sections, was also evaluated for MCT and CD147 expression. DCIS was found in 45 sections, and results for MCT and CD147 expression were always concordant with the corresponding invasive lesion.

Biological and clinical data available allowed the assessment of correlations with MCT and CD147 expression (Table 3). Importantly, we found significant correlations between prognostic parameters and both MCT and CD147 membranous expression. Correlations between MCT1 expression and basal-like subtype ($P < 0.0001$), high histological grade ($P = 0.0003$), absence of ER and PR expression ($P < 0.0001$ and $P = 0.0001$, respectively), CK5 and CK14 expression ($P = 0.0362$ and $P = 0.0006$, respectively), vimentin expression ($P < 0.0001$) and Ki67 expression ($P = 0.0297$) were observed. No association was observed between MCT4 expression and clinicopathological

Table 1. Frequency of monocarboxylate transporter (MCT) 1, MCT4 and CD147 expression in breast carcinoma tissues compared with normal breast epithelium

	n	Positive (%)	*P*
MCT1			
Normal breast epithelium	53	3 (5.7)	**0.0138**
Breast carcinoma	221	43 (19.4)	
MCT4			
Normal breast epithelium	45	0 (0.0)	0.0823
Breast carcinoma	219	16 (7.3)	
CD147			
Normal breast epithelium	47	2 (4.2)	0.2570
Breast carcinoma	219	24 (11.0)	

Significant values are shown in bold.

Table 2. Association between CD147 and monocarboxylate transporter (MCT) expression in breast carcinoma samples

	n	CD147 Positive (%)	*P*
MCT1			
Negative	172	5 (2.9)	**<0.0001**
Positive	42	19 (45.2)	
MCT4			
Negative	200	19 (9.5)	**0.0083**
Positive	16	5 (31.2)	

Significant values are shown in bold.

Table 3. Associations of monocarboxylate transporter (MCT) 1, MCT4 and CD147 expression with clinicopathological data from breast cancer cases

Clinicopathological data	MCT1			MCT4			CD147		
	n	Positive (%)	*P*	*n*	Positive (%)	*P*	*n*	Positive (%)	*P*
Tumour size, mm									
<20	73	10 (13.7)	0.2544	72	6 (8.3)	0.3152	73	7 (9.6)	0.7334
20–50	105	21 (20.0)		104	9 (8.6)		104	13 (12.5)	
>50	25	7 (28.0)		25	0 (0.0)		25	2 (8.0)	
Subtype									
Luminal	125	13 (10.4)	**<0.0001**	122	10 (8.2)	0.9529	123	5 (4.1)	**<0.0001**
Basal-like	42	21 (50.0)		42	3 (7.1)		42	14 (33.3)	
HER2 overexpressing	32	5 (15.6)		33	3 (9.1)		32	4 (12.5)	
Histological grade									
I	45	5 (11.1)	**0.0003**	43	5 (11.6)	0.1637	44	0 (0.0)	**<0.0001**
II	104	13 (12.5)		103	4 (3.9)		102	6 (5.9)	
III	71	25 (35.2)		71	7 (9.8)		71	18 (25.4)	
Oestrogen receptor									
Negative	95	30 (31.6)	**<0.0001**	95	6 (6.3)	0.5989	93	19 (20.4)	**0.0002**
Positive	125	13 (10.4)		122	10 (8.2)		123	5 (4.1)	
Progesterone receptor									
Negative	137	38 (27.7)	**0.0001**	139	11 (7.9)	0.6843	136	22 (16.2)	**0.0014**
Positive	80	5 (6.2)		78	5 (6.4)		80	2 (2.5)	
HER2 overexpression									
Negative	175	37 (21.1)	0.2135	172	11 (6.4)	0.4959	174	19 (10.9)	>0.9999
Positive	40	5 (12.5)		41	4 (9.8)		39	4 (10.2)	
EGFR									
Negative	206	39 (18.9)	0.2343	204	14 (6.9)	0.1925	204	21 (10.3)	0.1108
Positive	11	4 (36.4)		11	2 (18.2)		11	3 (27.3)	
CK5									
Negative	174	29 (16.7)	**0.0362**	171	10 (5.8)	0.0974	170	14 (8.2)	**0.0116**
Positive	46	14 (30.4)		46	6 (13.0)		47	10 (21.3)	
CK14									
Negative	201	34 (16.9)	**0.0006**	199	15 (7.5)	>0.9999	199	17 (8.5)	**<0.0001**
Positive	15	8 (53.3)		15	1 (6.7)		15	7 (46.7)	
Vimentin									
Negative	175	26 (14.8)	**<0.0001**	173	12 (6.9)	0.4869	172	9 (5.2)	**<0.0001**
Positive	36	17 (47.2)		36	4 (11.1)		36	15 (41.7)	

Table 3. (*Continued*)

Clinicopathological data	MCT1			MCT4			CD147		
	n	Positive (%)	*P*	*n*	Positive (%)	*P*	*n*	Positive (%)	*P*
Ki67									
<20%	139	21 (15.1)	**0.0297**	138	9 (6.5)	0.5258	138	10 (7.2)	**0.0179**
>20%	81	22 (27.2)		79	7 (8.9)		79	14 (17.7)	
Lymph node metastasis									
Absent	95	21 (22.1)	0.2771	92	7 (7.6)	0.8866	95	15 (15.8)	0.1566
Present	100	16 (16.0)		99	7 (7.1)		99	9 (9.1)	

EGFR, Epidermal growth factor receptor; CK, cytokeratin.
Significant values are shown in bold.

factors. CD147 expression associations were very similar to those of MCT1: CD147 correlated with basal-like subtype ($P < 0.0001$), high histological grade ($P < 0.0001$), absence of ER and PR expression ($P = 0.0002$ and $P = 0.0014$, respectively), CK5 and CK14 expression ($P = 0.0116$ and $P < 0.0001$, respectively), vimentin expression ($P < 0.0001$) and Ki67 expression ($P = 0.0179$).

When analysing MCT1/CD147 co-expression (data not shown) as an indicator of transporter activity, we observed the same associations as for MCT1 and CD147 alone. The clinicopathological significance of MCT4/CD147 co-expression was not assessed since the number of cases co-expressing these two molecules was very low ($n = 5$).

Kaplan–Meier survival curves were estimated for each marker (MCTs and CD147), but no significant correlations were found (data not shown).

Discussion

Some evidence points to MCTs as potential targets for cancer therapy.[19,22,37] However, the role of these proteins in solid tumour development and survival is still unclear, especially in breast cancer, where there is a very limited number of studies.

A previous report on breast cancer has suggested a possible silencing of MCT1 expression.[26] However, in the present study we showed a significant gain in MCT1 plasma membrane expression and no significant alteration in MCT4 expression in tumours when compared with non-neoplastic tissue. Considering the metabolic alterations of cancer, where high glycolytic rates lead to high levels of lactate, an increase in MCT1 expression would allow continuous glycolysis, avoiding intracellular acidosis and subsequent apoptosis. Also, and importantly, this would give a powerful growth advantage to cancer cells, necessary for progression to invasiveness.[38] Notably, and besides the increased expression in tumour cells, the presence of MCT1 in the plasma membrane was associated with various clinical and biological parameters. Importantly, MCT1 expression correlated with a basal-like phenotype, and, as expected, was associated with most of the basal markers studied, such as CK5, CK14 and vimentin, and inversely associated with the expression of ER and PR. These results are supported by data available in expression array datasets (ONCOMINE),[39] where increased expression in MCT1 can be seen in basal-like breast carcinoma.[40,41] Other correlations, such as the association with Ki67 proliferative index and high histological grade, suggest that MCT1 is present in more aggressive tumours.

Taking into consideration the need to export lactate in cancer cells and the fact that MCT4 is a low-affinity transporter found in highly glycolytic tissues,[42] an increase in MCT4 expression would be expected in breast tumours, similar to that observed in both colorectal[24] and cervical carcinomas.[23] However, in the present work, although MCT4 was not expressed in normal tissue and was detected in the plasma membrane of some tumour samples, this increase was not significant. Thus, our results point to preferential expression of the MCT isoform 1, as an adaptation to a malignant phenotype. Actually, despite the similar physiological function of MCT1 and MCT4, they are regulated by different mechanisms; for example, it has recently been described that hypoxia-inducible factor 1α, a major regulator of tumour cell adaptation to hypoxic stress, up-regulates MCT4, but not MCT1.[43]

CD147, a protein that, among many other functions,[27–29] stimulates synthesis of matrix metalloproteinases, is up-regulated in many human

cancers,[27,28,44,45] including breast carcinomas,[44,45] where it is described as a prognostic factor.[44] In the present work, this documented increase in CD147 was not observed, but the close association with both MCT1 and MCT4 previously demonstrated[25,30–35] was confirmed in the breast tumour samples analysed here. As observed for MCT1, CD147 expression was more frequently found in basal-like, ER– and PR– and high histological grade tumours as well as in tumours expressing CK5, CK14, vimentin and Ki67, which is in accordance with previous results on CD147 expression in breast carcinoma.[44] MCT1/CD147 co-expression showed similar associations with MCT1 and CD147 alone. A similar importance for MCT1/CD147 co-expression in gastric cancer has already been described by our group in gastric cancer, where we observed an association of MCT1/CD147 co-expression with indicators of worse prognosis, such as advanced gastric cancer, higher TNM staging and presence of lymph node metastasis.[25] However, although these associations may be mostly attributed to CD147 and its many functions in cancer,[27–29] MCT1 alone also appears to have an important role in tumours with worse prognosis, as evidenced by the significant results obtained. Importantly, it should be noted that less than half of cases expressing MCT1 and MCT4 on the plasma membrane co-express CD147. This suggests that MCT trafficking to the plasma membrane might be dependent on another ancillary protein, as pointed out by recent *in vitro* evidence.[46]

In the present study, we have investigated the expression of MCT isoforms 1 and 4, and their chaperone CD147. Most importantly, we have evaluated for the first time the correlation between MCT expression in breast cancer and the clinicopathological data. It is important to highlight that MCT1 is up-regulated in a subset of aggressive breast carcinomas (basal-like) and, since these tumours do not have a specific molecular therapy,[8] the development of therapeutic approaches targeting MCT1 could be a promising strategy to treat such tumours. However, other studies, including *in vitro* approaches are warranted to confirm these observations.

Acknowledgements

C.P. and A.A. received PhD fellowships from FCT (Portuguese Foundation for Science and Technology, SFRH/BD/27465/2006 and SFRH/BD/15316/2005, respectively). We also thank Dr Vitor Carneiro (pathologist from the Hospital do Divino Espírito Santo, Ponta Delgada, São Miguel, Açores) for his help in retrieving the breast cancer samples for this study.

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PUBLICATIONS

Paper 7

BMC Cancer

RESEARCH ARTICLE Open Access

Alterations in Vitamin D signalling and metabolic pathways in breast cancer progression: a study of VDR, CYP27B1 and CYP24A1 expression in benign and malignant breast lesions Vitamin D pathways unbalanced in breast lesions

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Abstract

Background: Breast cancer is a heterogeneous disease associated with different patient prognosis and responses to therapy. Vitamin D has been emerging as a potential treatment for cancer, as it has been demonstrated that it modulates proliferation, apoptosis, invasion and metastasis, among others. It acts mostly through the Vitamin D receptor (VDR) and the synthesis and degradation of this hormone are regulated by the enzymes CYP27B1 and CYP24A1, respectively. We aimed to study the expression of these three proteins by immunohistochemistry in a series of breast lesions.

Methods: We have used a cohort comprising normal breast, benign mammary lesions, carcinomas *in situ* and invasive carcinomas and assessed the expression of the VDR, CYP27B1 and CYP24A1 by immunohistochemistry.

Results: The results that we have obtained show that all proteins are expressed in the various breast tissues, although at different amounts. The VDR was frequently expressed in benign lesions (93.5%) and its levels of expression were diminished in invasive tumours (56.2%). Additionally, the VDR was strongly associated with the oestrogen receptor positivity in breast carcinomas. CYP27B1 expression is slightly lower in invasive carcinomas (44.6%) than in benign lesions (55.8%). In contrast, CYP24A1 expression was augmented in carcinomas (56.0% in *in situ* and 53.7% in invasive carcinomas) when compared with that in benign lesions (19.0%).

Conclusions: From this study, we conclude that there is a deregulation of the Vitamin D signalling and metabolic pathways in breast cancer, favouring tumour progression. Thus, during mammary malignant transformation, tumour cells lose their ability to synthesize the active form of Vitamin D and respond to VDR-mediated Vitamin D effects, while increasing their ability to degrade this hormone.

Background

Breast cancer is one of the major causes of death by cancer in women worldwide [1]. Nowadays, breast cancer is no longer considered to be a single disease, but is rather comprised of distinct tumour subtypes displaying different clinical outcomes [2]. Over the lifetime of the individual, in order to a tumour to develop it needs a combination of low-penetrance genetic factors and environmental aspects. Ultimately, cancer results from alterations in the control of the complex balance of proliferation, differentiation and programmed cell death [3] and these processes appear to be regulated by intrinsic and extrinsic factors, like niche signals, hormonal and dietary aspects, among others [4], [5].

Vitamin D is a lipid soluble substance that belongs to the family of secosteroid hormones. Its physiological

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role has been classically associated with calcium regulation and phosphate transport in bone metabolism. Apart from this endocrine role, subsequent studies have widened the range of functions for Vitamin D and this has been particularly important in the field of cancer research. Several authors have demonstrated, in various models of cancer (including the breast), the ability of Vitamin D to perform autocrine and paracrine functions. Specifically, it has been demonstrated the capacity to modulate cancer features, namely proliferation and differentiation [6], apoptosis [7], angiogenesis [8], invasion and metastasis [9].

Vitamin D exerts most of its biological activities by binding to a specific high-affinity receptor, the Vitamin D Receptor (VDR), that was first identified in a breast cancer cell line in 1979 [10]. The VDR belongs to the superfamily of nuclear receptors for steroid hormones and regulates gene expression by acting as a ligand-activated transcription factor [11]. Several studies have demonstrated that the VDR knockout mice display a higher incidence rate of carcinogen-induced preneoplastic breast lesions when compared with their littermates [12], [13]. These reports highlight the importance of the VDR deficiency in sensitizing the mammary gland to transformation in response to a carcinogenic agent. Immunohistochemical studies have confirmed that the VDR is expressed in samples from normal breast tissues [14] and also in breast cancer biopsy specimens [15]. Because the VDR is expressed in the mammary gland and Vitamin D has been shown to display anticarcinogenic properties, this hormone has emerged as a promising targeted therapy. But in order to keep the homeostasis of the organism the amount of circulating Vitamin D has to be tightly regulated. This is a very complex process, in which the main components are the enzymes 1α-hydroxylase/CYP27B1 (encoded by the gene *CYP27B1*) and 24-hydroxylase/CYP24A1 (encoded by the gene *CYP24A1*). CYP27B1 is responsible for the synthesis of the biologically active form of Vitamin D (1,25-dihydroxyvitamin D), whereas CYP24A1 mediates the catabolism of Vitamin D [16]. Several studies have focused their attention in the comparison of the levels of these enzymes in normal and tumour tissue. It has been observed that both CYP27B1 and CYP24A1 are up-regulated in breast tumours when compared with normal tissue. However, deregulated expression of CYP24A1 seems to abrogate the effects of CYP27B1, resulting in the degradation of Vitamin D to less active metabolites [17]. In contrast, a recent paper has demonstrated that CYP27B1 mRNA in breast tumours is decreased in comparison with normal mammary tissue [18]. Despite these findings, no reports regarding the expression by immunohistochemistry of the VDR, CYP27B1 and CYP24A1 in the mammary gland have been described. The main purpose of this work was to perform an immunohistochemical study of the expression of the VDR, CYP27B1 and CYP24A1 in a comprehensive series of human breast tissues comprised of normal breast, benign mammary lesions, carcinomas *in situ* and invasive breast carcinomas.

Methods

Patient's selection and Tissue Microarray construction

We have studied a cohort of 379 benign lesion samples and 189 cases of carcinomas *in situ*, collected from the archives of the Pathology Department of General Hospital of UNIMED in Araçatuba, Brazil. Three hundred and fifty cases of invasive breast carcinomas were retrieved from the archives of the Pathology Department of the Federal University of Santa Catarina, Florianópolis, Brazil (161 cases) and from the Pathology Department of General Hospital of UNIMED in Araçatuba, Brazil (189 tumour samples). This last series of 189 invasive carcinomas contains, in the same block, the aforementioned carcinomas *in situ*. Additionally, 29 cases of normal breast tissue were included in the study. The normal breast tissue, carcinomas *in situ* and invasive tumour samples were collected between 1994 and 2004. The series of benign lesions was collected between 2002 and 2006.

Representative areas of the different lesions were carefully selected on the H&E-stained sections, by 2 pathologists (DV and LAV) and marked on individual paraffin blocks. Two tissue cores (2 mm in diameter) were obtained from each selected specimen and precisely deposited into a recipient paraffin block using a TMA (Tissue Microarray) workstation (TMA builder, LabVision Corporation, USA). Several TMA blocks were constructed (40 for the invasive breast carcinomas, 22 for the carcinomas *in situ* and 17 for the benign lesions), each containing 24 tissue cores, arranged in a 4×6 sector. In each TMA block, at least 3 nonneoplastic breast tissue cores were also included as controls and 1 core of a non-breast sample (we have used testicular and liver tissues). To homogenize the paraffin of the receptor block and the paraffin of the cores extracted from the donor blocks, the TMAs were kept at 37°C for 3 hours. After construction, 2-μm tissue sections were cut and adhered to Superfrost Plus glass slides. An H&E-stained section from each block was reviewed to confirm the presence of morphological representative areas of the original lesions.

The present study has been conducted under the national regulative law for the usage of biological specimens from tumour banks, where the samples are exclusively available for research purposes in the case of retrospective studies.

Immunohistochemistry

Immunohistochemical staining for Oestrogen Receptor (ER), HER2 and CK5 (Cytokeratin 5) was performed

using the streptavidin-biotin-peroxidase technique (LabVision Corporation) in each set of glass slides comprising the TMAs, whereas P-cadherin (P-cad), EGFR (Epidermal Growth Factor Receptor) and Progesterone Receptor (PgR) used the HRP labelled polymer (Dako-Cytomation, USA) as described elsewhere [19]. Antigen unmasking for VDR was performed using a solution of pepsin A (4 g/L; Sigma-Aldrich) for 30 minutes at 37°C. Epitope retrieval for CYP27B1 and CYP24A1 was performed using a dilution of 1:100 of citrate buffer, pH = 6.0 (Vector Laboratories, Burlingame, CA, USA) at 98°C for 30 minutes. The antigen retrieval times, antibodies, dilutions and suppliers are listed in Table 1. Primary antibody incubation was performed overnight at 4°C for VDR and CYP24A1 and for 1 h at room temperature for CYP27B1. After washes, the slides were incubated with secondary antibody associated with HRP labelled polymer (ImmunoLogic, The Netherlands) for VDR or incubated with biotinylated secondary antibody (Santa Cruz, USA) followed by streptavidin-conjugated peroxidase (Labvision) during 15 min for CYP24A1 and CYP27B1, and immediately revealed with DAB (Dako-Cytomation). Tissues were then counterstained with Mayer's haematoxylin, dehydrated and cover-slipped using a permanent mounting solution (Zymed, USA). Positive and negative controls were included in each run in order to guarantee the reliability of the assays. Paraffin sections of a basal cell carcinoma of the skin, normal colon and normal liver were used as positive controls for VDR, CYP27B1 and CYP24A1 expression, respectively.

Scoring and statistical analysis

The evaluation of the immunohistochemical results was performed by three pathologists (FS, FM and LAV). VDR nuclear expression was evaluated using the H-score method: intensity ranked from 1 to 3 (1 - weak, 2 - moderate, 3 - strong), and extension ranked from 1 to 10 (1 - 0-10% cells, 2 - 11-20% cells and so on, until a maximum score of 10) [20]. The scores for intensity and extension were multiplied and the following criterion was applied: the cases were considered negative when ranging from 1 to 4; samples ranking from 5 to 30 were considered to be positive. Considering the lack of previous reports for the immunohistochemical evaluation of the CYP27B1 and CYP24A1, we considered the cases to be positive only when cytoplasmic staining was observed. The other markers were scored as described in previous studies from our group [19], [21].

The Statview 5.0 software package (SAS Institute, USA) was used for all statistical analysis. Correlations between discrete variables were performed using the chi-square test and analysis of variance was employed to search for associations between continuous and discrete variables. In all analyses, a p value < 0.05 was considered to be statistically significant.

Cell culture and Western blotting

MDA-MB-231 breast cancer cells were grown in complete Dulbecco's Modified Eagle Medium (DMEM) in the presence of 10% foetal bovine serum (Invitrogen, USA). Treatments with Vitamin D 100 nM (Cayman Chemical, USA) and ethanol (vehicle) were performed for 72 h, while the treatment with PTH (Parathyroid Hormone) (Sigma-Aldrich, Germany) 100 nM and water (vehicle) were performed for 4 h. Total cell lysates were obtained and the samples were separated in an SDS-polyacrylamide gel. After blotting into a nitrocellulose membrane (GE Healthcare Life Sciences, UK), staining for CYP27B1 and CYP24A1 was performed using the antibodies (Santa Cruz, USA) presented on Table 1 overnight at a dilution of 1:200. After washes, the membranes were incubated with a mouse anti-goat HRP secondary antibody (Santa Cruz) and were revealed with ECL (GE Healthcare Life Sciences).

RNA extraction and Real-time PCR

RNA was extracted from formalin-fixed paraffin-embedded breast lesions using the RecoverAll Total Nucleic Acid Isolation Kit (Ambion, USA), according to the manufacturer's protocol. After extraction, RNA was quantified using NanoDrop spectrophotometer (Thermo Scientific, USA). cDNA was synthesized using the Omniscript Reverse Transcription kit (Qiagen, Germany) following the manufacturer's instructions. Finally, real-time PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems, USA), using 2 mL of cDNA and in accordance to the manufacturer's protocol. The TaqMan Gene Expression Assays used were Hs00172113_m1 (VDR), Hs00168017_m1 (CYP27B1) and Hs00167999_m1 (CYP24A1). Reactions were performed using standard cycle parameters on an ABI PRISM Sequence 7000 Detection System (Applied

Table 1 Sources and dilutions of primary antibodies related to the Vitamin D metabolism used in this study for immunohistochemistry

Antibody	Clone	Manufacturer	Time of incubation (min)	Dilution	Antigen retrieval (min)
VDR	9A7γE10.4	Calbiochem, Germany	overnight	1:50	30
CYP27B1	C12	Santa Cruz, USA	60	1:200	30
CYP24A1	C18	Santa Cruz, USA	overnight	1:75	30

Biosystems). Relative transcript levels were determined using Human GAPDH Endogenous Control (Applied Biosystems) as an internal reference. Differences between the breast tissue samples were determined using comparative delta C_T method [22]. All reactions were done in triplicate and expressed as mean of the values from three separate experiments.

Results

VDR, CYP27B1 and CYP24A1 immunohistochemical staining

The expression patterns of the VDR, CYP27B1 and CYP24A1 have been evaluated by immunohistochemistry in 947 breast tissue samples arranged in 79 TMAs. From this set of cases, some samples could not be assessed due to the fact that either the core had fallen out or it did not have enough biological material to study. In all TMAs, positive and negative cases were obtained for each protein. The immunostainings for these markers had been previously validated in whole tissue sections with an overall agreement of 90%. A panel with representative immunostainings for each protein in different breast tissues is shown in Figure 1. We have observed that the VDR displays nuclear staining, as would be expected from a nuclear receptor which acts as a transcription factor. Considering CYP27B1 and CYP24A1 expression, nothing has ever been described on their expression status in the mammary gland, as far as we know. This is the first report showing the expression of these two enzymes in breast lesions. These proteins present cytoplasmic and granular staining, which could reflect their mitochondrial localisation. All proteins (VDR, CYP27B1 and CYP24A1) have been found to be expressed in all lesions studied and also in the normal breast tissue, although at different levels.

The differential expression of CYP27B1 and CYP24A1 was technically validated. MDA-MB-231 breast cancer cells have been treated with PTH 100 nM and Vitamin D 100 nM and total cell lysates have been extracted. Western blotting analysis has confirmed the expression of CYP27B1 and CYP24A1 upon treatment with the aforementioned hormones (Additional file 1: Figure S1). Additionally, using a group of randomly selected tissue samples, RNA was isolated and used in real-time PCR to confirm the immunohistochemical results (Additional file 2: Table S1). Our results have shown that positive cases in the TMAs displayed cDNA amplification in the real-time PCR and the opposite situation was observed for cases where no staining was present in the TMAs.

Expression of the VDR, CYP27B1 and CYP24A1 in benign lesions of the mammary gland

In order to study the VDR, CYP27B1 and CYP24A1 expression in benign lesions of the mammary gland, we have evaluated 379 cases arranged in 17 TMAs. The series consisted of a variety of breast lesions, namely usual and atypical ductal hyperplasias (UDH represent 20.1%, corresponding to 76 samples; while ADH represent 5.4%, corresponding to 21 samples), columnar cell lesions (CCL - 25.6% of cases, corresponding to 97 samples), papillomatosis (16.9% of cases, corresponding to 64 samples) and adenosis (17.2% of cases, corresponding to 65 samples). The percentage of immunoreactive cases for the VDR was very high (93.5%, corresponding to 259 cases out of 277). Regarding the expression of CYP27B1, we have observed 55.8% of positive cases, corresponding to 173 lesions out of 310. Concerning CYP24A1 expression, we have detected 62 positive cases out of 327 samples (19.0%). Amongst all lesions, ADH cases were overall less immunoreactive to the three proteins.

We have correlated the histological classification of the benign lesions with the VDR, CYP27B1 and CYP24A1 expression, but no significant associations have been found (see Table 2 for further details).

Expression of the VDR, CYP27B1 and CYP24A1 in breast carcinomas *in situ*

A fully characterized series of 189 breast carcinomas *in situ* arranged in 22 TMAs was assessed for the expression patterns of VDR, CYP27B1 and CYP24A1. For the VDR, we have observed that 62 cases out of 131 cases (47.3%) displayed staining for this protein. Concerning CYP27B1 expression, we have encountered positive staining in 66.4% of the cases (91 out of 137 samples); whereas CYP24A1 expression was observed in 56.0% of the tumours (70 out of 125 cases).

We have also assessed the expression of other breast cancer biomarkers in our cohort (ER, HER2 and PgR and basal markers as defined by our group [19] and others [23]) and looked for the existence of correlations between the expression of the Vitamin D partners and these molecular markers (Table 3). ER expression has been observed in 117 cases (61.9%), HER2 protein was present in 37 cases (15.6%) and PgR expression was detected in 90 cases (47.6%). We have also tested our series for basal markers and have obtained the following results: EGFR expression is present in 10 cases (5.3%), CK5 is positive in 15 cases (7.9%) and P-cadherin was observed in 36 samples (19.0%). Expression of the VDR correlated positively with ER status ($p = 0.0227$), with a higher percentage of VDR-positive cases among the ER-positive tumours - 74.2% (46 out of 62 cases). Additionally, we have seen that there is an inverse correlation between the expression of the VDR and P-cadherin ($p = 0.0078$). CYP27B1 expression only presented an inverse correlation ($p = 0.0295$) with EGFR expression, but the number of cases positive for EGFR was very low. No statistically significant associations have been observed between CYP24A1 expression and the markers studied.

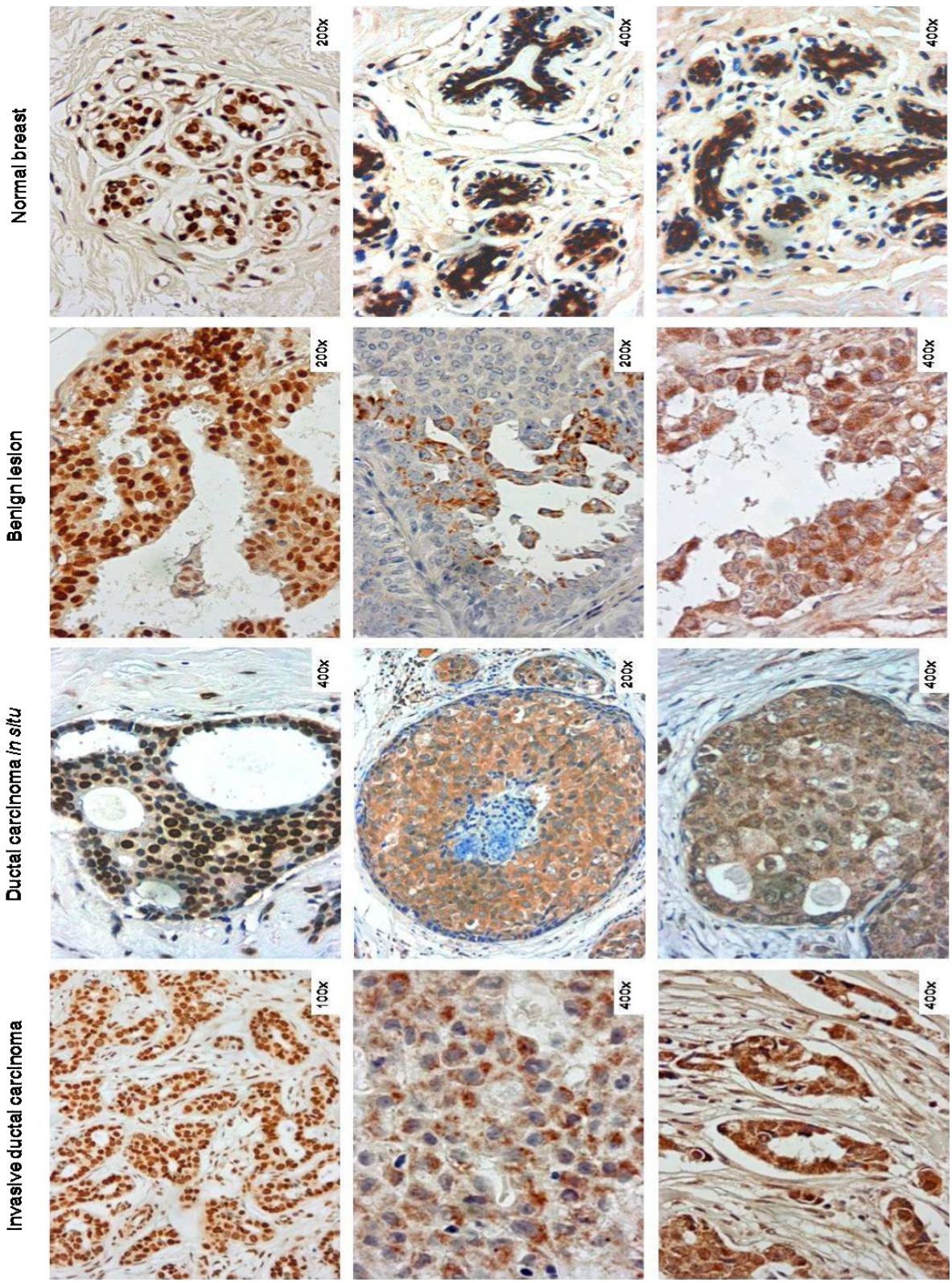

Figure 1 Immunohistochemical staining for the VDR, CYP27B1 and CYP24A1 in the different types of breast tissue

Table 2 VDR, CYP27B1 and CYP24A1 expression in the various types of benign breast lesions

	VDR		CYP27B1		CYP24A1	
	+ (%)	- (%)	+ (%)	- (%)	+ (%)	- (%)
Usual ductal hyperplasia	84 (92.3)	7 (7.7)	57 (55.9)	45 (44.1)	23 (20.5)	89 (79.5)
Atypical ductal hyperplasia	9 (81.8)	2 (18.2)	4 (36.4)	7 (63.6)	1 (7.1)	13 (92.9)
Columnar cell lesions	63 (95.5)	3 (4.5)	43 (55.8)	34 (44.2)	13 (16.5)	66 (83.5)
Papillomatosis	45 (95.7)	2 (4.3)	30 (56.6)	23 (43.4)	9 (17.0)	44 (83.0)
Adenosis	49 (92.5)	4 (7.5)	32 (55.2)	26 (44.8)	13 (22.0)	46 (78)
p value	**0.4847**		**0.7994**		**0.6842**	

Expression of the VDR, CYP27B1 and CYP24A1 in invasive mammary carcinomas

We have evaluated 350 cases of invasive breast carcinomas arranged in 40 TMAs. The cohort corresponds to 189 cases of the series for which there was an *in situ* component in the adjacent area of the invasive tumour and an additional series of 161 cases of invasive breast carcinomas. Positive staining for the VDR has been observed in 56.2% of the cases (172 out of 306 cases). Regarding CYP27B1 expression, 44.6% of cases were positive (123 out of 276 samples), whereas 53.7% of cases (151 out of 281 tumours) presented positivity for CYP24A1.

Next, we searched for associations between the expression of Vitamin D partners and the expression of the molecular markers mentioned in the previous section (Table 4). We have obtained 197 cases (56.3%) positive for ER, 70 cases (20%) for HER2 and 143 cases (40.9%) for PgR. As for basal markers, we have observed that 13 cases (3.7%) were positive for EGFR expression, 48 cases (13.7%) presented positivity for CK5 and 93 cases (26.6%) stained for P-cadherin.

A statistically significant association was observed between the VDR-positive cases and ER-positive cases (p = 0.0002). Additionally, VDR-positive cases have also been significantly correlated with HER2-negative cases (p = 0.0238), but this is probably due to the low number of positive cases for HER2 in our series of mammary carcinomas. CYP27B1 expression presented no significant associations with any of the markers analyzed. PgR was the only marker that displayed an inverse correlation with CYP24A1: specifically, cases positive for PgR were mostly negative for CYP24A1 (p = 0.0485).

The series of 189 tumours with both components (carcinomas *in situ* and the corresponding invasive tumour) allowed the evaluation of the expression of the VDR, CYP27B1 and CYP24A1 simultaneously in the

Table 3 VDR, CYP27B1 and CYP24A1 and other breast cancer biomarkers expression in carcinomas *in situ*

		VDR		CYP27B1		CYP24A1	
		+ (%)	- (%)	+ (%)	- (%)	+ (%)	- (%)
ER	+ (%)	46 (35.1)	38 (29.0)	58 (42.3)	29 (21.2)	41 (32.8)	36 (28.8)
	- (%)	16 (12.2)	31 (23.7)	33 (24.1)	17 (12.4)	29 (23.2)	19 (15.2)
	p value	**0.0227**		**ns**		**ns**	
HER2	+ (%)	9 (6.9)	14 (10.7)	18 (13.1)	7 (5.1)	9 (7.2)	12 (9.6)
	- (%)	53 (40.5)	55 (42.0)	73 (53.3)	39 (28.5)	61 (48.8)	43 (34.4)
	p value	**ns**		**ns**		**ns**	
PgR	+ (%)	35 (26.7)	30 (22.9)	49 (35.8)	18 (13.1)	38 (30.4)	22 (17.6)
	- (%)	27 (20.6)	39 (29.8)	42 (30.7)	28 (20.4)	32 (25.6)	33 (26.4)
	p value	**ns**		**ns**		**ns**	
CK5	+ (%)	3 (2.3)	8 (6.1)	7 (5.1)	4 (2.9)	8 (6.4)	4 (3.2)
	- (%)	59 (45.0)	61 (46.6)	84 (61.3)	42 (30.7)	62 (49.6)	51 (40.8)
	p value	**ns**		**ns**		**ns**	
EGFR	+ (%)	1 (0.8)	5 (3.8)	2 (1.5)	5 (3.7)	5 (4.0)	3 (2.4)
	- (%)	61 (46.6)	64 (48.9)	89 (65.0)	41 (29.9)	65 (52.0)	52 (41.6)
	p value	**ns**		**0.0295**		**ns**	
P-cad	+ (%)	4 (3.1)	16 (12.2)	14 (10.2)	12 (8.8)	16 (12.8)	7 (5.6)
	- (%)	58 (44.3)	53 (40.5)	77 (56.2)	34 (24.8)	54 (43.2)	48 (38.4)
	p value	**0.0078**		**ns**		**ns**	

ns: not significant.

Table 4 VDR, CYP27B1 and CYP24A1 and other breast cancer biomarkers expression in invasive breast tumours

		VDR		CYP27B1		CYP24A1	
		+ (%)	- (%)	+ (%)	- (%)	+ (%)	- (%)
ER	+ (%)	114 (37.3)	60 (19.6)	70 (25.4)	86 (31.2)	93 (33.1)	66 (23.5)
	- (%)	58 (19.0)	74 (24.2)	53 (19.2)	67 (24.3)	58 (20.6)	64 (22.8)
	p value	**0.0002**		**ns**		**ns**	
HER2	+ (%)	26 (8.6)	34 (11.3)	31 (11.4)	25 (9.2)	29 (10.4)	30 (10.8)
	- (%)	144 (47.7)	98 (32.5)	90 (33.1)	126 (46.3)	121 (43.5)	98 (35.3)
	p value	**0.0238**		**ns**		**ns**	
PgR	+ (%)	71 (23.3)	59 (19.3)	52 (18.8)	64 (23.2)	71 (25.3)	46 (16.4)
	- (%)	100 (32.8)	75 (24.6)	71 (25.7)	89 (32.2)	80 (28.5)	84 (29.9)
	p value	**ns**		**ns**		**0.0485**	
CK5	+ (%)	27 (8.8)	19 (6.2)	15 (5.4)	24 (8.7)	27 (9.6)	16 (5.7)
	- (%)	145 (47.4)	115 (37.6)	108 (39.1)	129 (46.7)	124 (44.1)	114 (40.6)
	p value	**ns**		**ns**		**ns**	
EGFR	+ (%)	4 (1.3)	7 (2.3)	4 (1.5)	6 (2.2)	6 (2.1)	3 (1.1)
	- (%)	166 (54.8)	126 (41.6)	118 (43.1)	146 (53.3)	145 (51.8)	126 (45.0)
	p value	**ns**		**ns**		**ns**	
P-cad	+ (%)	42 (13.8)	40 (13.1)	30 (10.9)	42 (15.2)	40 (14.3)	37 (13.2)
	- (%)	129 (42.3)	94 (30.8)	93 (33.7)	111 (40.2)	110 (39.3)	93 (33.2)
	p value	**ns**		**ns**		**ns**	

ns: not significant.

two types of tumours (Additional file 2: Table S2). The results obtained show that the three proteins (VDR, CYP27B1 and CYP24A1) display a statistically significant correlation of expression between the two sections (carcinomas *in situ* and the matching invasive tumour). Thus, positive cases in the *in situ* component are also positive in the invasive component and the same is observed for the negative cases.

Expression of the VDR, CYP27B1 and CYP24A1 according to the type of breast lesion

The frequencies of protein expression of the VDR, CYP27B1 and CYP24A1 in the different mammary tissues are shown in Figure 2. The normal mammary gland (29 cases), as expected, is positive for the expression of the VDR in all the cases studied (100%). The majority of the samples also displays immunostaining for CYP27B1 (63.6%) and, in contrast, the levels of expression of CYP24A1 are low (29.6%). The VDR is also highly expressed in benign lesions (93.5%) with a reduction in the percentage of positive cases in carcinomas *in situ* (47.3%) and in invasive carcinomas (56.2%). CYP27B1 expression does not vary greatly between the different breast lesions. However, between *in situ* and invasive carcinomas, a statistically significant decrease in the percentage of positive cases was observed (from 66.4% in carcinomas *in situ* to 44.6% in invasive carcinomas). In contrast, the expression of CYP24A1 is increased in carcinomas (56.0% in carcinomas *in situ* and 53.7% in invasive carcinomas) compared with the benign lesions (19.0%), which are mostly negative.

Discussion

Vitamin D mediates anti-proliferative and pro-differentiation signalling in various epithelial tissues, including

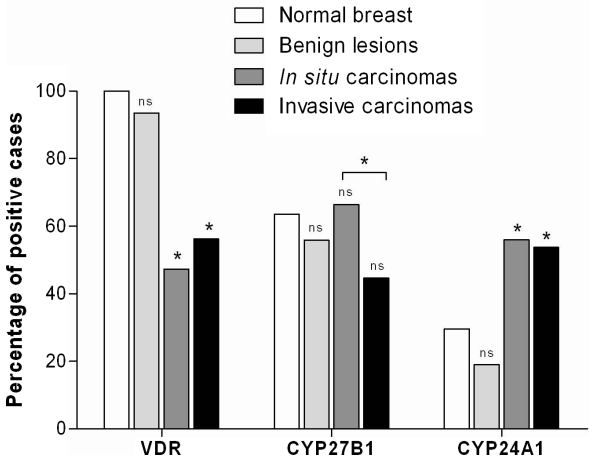

Figure 2 Percentage of positive cases for VDR, CYP27B1 and CYP24A1 in the various types of breast samples studied. Statistical analysis shown use normal breast as reference. An additional result is presented comparing the number of CYP27B1 positive cases between *in situ* and invasive carcinomas. (ns - not significant; * $p < 0.05$).

the mammary gland [6]. Therefore, it is reasonable to assume that disruption of the Vitamin D signalling and metabolic pathways may occur during tumour development. To explore this hypothesis, we have evaluated a cohort of 947 samples of human breast tissues for the presence of VDR, CYP27B1 and CYP24A1. Specifically, our series consisted of normal breast tissue (29 cases), preneoplastic benign mammary lesions (379 cases), carcinomas *in situ* (189 cases) and invasive breast carcinomas (350 cases). To the best of our knowledge, this is the first time that the expression of the VDR, CYP27B1 and CYP24A1 has been evaluated in histological sections of mammary lesions.

The three proteins have been found to be expressed in all breast tissues, although at different levels. VDR presented a nuclear localisation, as it would be expected for a nuclear receptor, while CYP27B1 and CYP24A1 enzymes displayed cytoplasmic staining with a granular pattern, which is consistent with their mitochondrial localisation. The immunohistochemical results were further validated and confirmed using quantitative real-time PCR and Western blotting.

Some studies have demonstrated that the VDR protein is expressed in samples from normal breast tissues and also in breast cancer biopsy specimens [14,15,24,25]. Our results have shown that the VDR is expressed in carcinomas. However, the percentage of positive cases that we have obtained (47.3% in carcinomas *in situ* and 56.2% in invasive carcinomas) is lower than the 80% to 90% that had been previously described in the literature [26,27]. This discrepancy can be explained by the development of new detection techniques and the use of different scoring methods. In this study, we have used the H-Score, the current method employed for other nuclear receptors, like ER [20], whereas in previous studies the presence of any staining was marked as positive. As far as we know, our study is the first to investigate the immunohistochemical expression of the VDR in a range of benign lesions and carcinomas *in situ* of the mammary gland. The percentage of positive cases for the VDR is higher in benign lesions than in invasive tumours (93.5% and 56.2%, respectively), while the carcinomas *in situ* display the lowest value of all (47.3%). There are some studies showing higher levels of VDR in tumour tissues [18,28], but this discrepancy can be attributed to the use of different evaluation techniques.

An interesting finding is the correlation between the expression of the VDR and the ER in both *in situ* and invasive carcinomas. In fact, the VDR is expressed in most ER-positive cases (54.7% in *in situ* carcinomas and 65.5% in invasive tumours). It is thought that one of the VDR functions is to counteract oestrogen-mediated proliferation and maintain differentiation [12]. Indeed, data support the concept that the anti-tumour effects of Vitamin D and its analogues on ER-positive human breast cancer cells are mediated through the down regulation of the ER itself and the attenuation of oestrogen responses, such as breast cancer cell growth [29,30]. Thus, being the VDR mostly expressed in ER-positive carcinomas, Vitamin D or its analogues may become an alternative therapy for these tumours in cases of resistance to ER-targeted therapy.

The levels of protein expression of CYP27B1 and CYP24A1 have not been previously studied in breast cancer. In colon cancer, a study using immunohistochemistry has demonstrated that CYP27B1 is present at equally high levels in normal colonic epithelium and colorectal cancer [31]. For CYP24A1 it has been shown that increasing amounts of this enzyme are present in normal colon tissue and pre-malignant lesions. In cancer, the expression of CYP24A1 decreases as a function of tumour cell dedifferentiation [32]. In breast tissues, McCarthy *et al.*[18] have demonstrated that CYP27B1 mRNA expression was significantly down regulated in adjacent non-cancerous tissue from women with breast cancer in comparison with individuals without cancer. Additionally, it has been shown that the expression of mRNA for CYP27B1 and the VDR was higher in carcinomas versus non-neoplastic tissue [17]. Considering differences in expression in benign and malignant breast tissues, we have observed an increased expression of CYP24A1 and a decreased expression of CYP27B1 with malignant progression. In fact, CYP27B1 was expressed in 55.8% of the preneoplastic lesions and this percentage is decreased in invasive tumours (44.6%), while carcinomas *in situ* display the highest value (66.4%) and these differences are statistically significant. In contrast, CYP24A1 is augmented more than 2.5 fold in invasive tumours (53.7%), compared with benign breast lesions (19.0%) and this difference is also significant ($p < 0.0001$). The *in situ* carcinomas exhibit the highest percentage of positive cases (56.0%). These observations are consistent with the results of Townsend and colleagues [17], which have demonstrated that there was an up regulation of CYP24A1 mRNA in breast tumour tissue, in comparison with normal breast. It has also been described that the *CYP24A1* gene is amplified in breast cancer [33]. In contrast, another study has found no differences in the expression of the VDR, CYP27B1 and CYP24A1 mRNA in breast cancer and non-neoplastic mammary tissue [34]. These contradictory results may be explained by recent reports where it is described that VDR and CYP24A1 are under the post-transcriptional control of miRNAs [35,36].

Breast cancer is a process that evolves through the accumulation of (epi)genetic events that drive uncontrolled proliferation and resistance to apoptosis. The active form of Vitamin D is known for its capacity to modulate proliferation and induce apoptosis [6]. Consequently, malignant cells would need to develop

mechanisms to deregulate Vitamin D metabolic and signalling pathways in order to allow tumour development [37]. Furthermore, it has been suggested that the Vitamin D produced in non-renal tissues is not released into the blood stream, but instead acts locally [38]. Therefore, the amount of Vitamin D available in the tissue depends on the relative amounts of CYP27B1 (synthesis) and CYP24A1 (catabolism). Accordingly, our results show a deregulation of these two enzymes in the different stages of breast carcinogenesis. The crucial step of transformation introduces a clear unbalance in the Vitamin D signalling and metabolic pathways. A reduction in the expression of the VDR in carcinomas indicates lower sensitivity of the tissue to Vitamin D control. Furthermore, a strong increase in CYP24A1 positive cases points to an enhanced ability of the cells to degrade this hormone. In contrast, the stable levels of CYP27B1 throughout the transformation process, with only a small decrease in invasive carcinomas, may reflect a lower capacity to metabolize Vitamin D into its active form.

Conclusions

In summary, this is the first study to report the expression of the VDR, CYP27B1 and CYP24A1 in a series of normal breast, preneoplastic mammary lesions, breast carcinomas *in situ* and invasive tumours. We have correlated the expression of these Vitamin D partners with the expression of a panel of tumour biomarkers. Furthermore, we have confirmed these results by real-time RT-PCR. Overall, our results on the expression of the VDR, CYP27B1 and CYP24A1 suggest that there is a deregulation of the Vitamin D metabolic and signalling pathways in breast cancer, in order to favour tumour progression. Thus, during breast malignant transformation, tumour cells lose their ability to synthesize the active form of Vitamin D and to respond to Vitamin D effects, while increasing their ability to degrade this hormone.

Additional material

Additional file 1: Figure S1: In MDA-MB-231 breast cancer cells CYP27B1 expression is induced by the treatment with PTH 100 nM for 4 h and CYP24A1 expression is induced by the treatment with Vitamin D ($1,25(OH)_2D_3$) 100 nM for 72 h. α-tubulin was used as a loading control

Additional file 2: Table S2: VDR, CYP27B1 and CYP24A1 expression in tumours that display both the *in situ* and the invasive component in the same histological section.

List of abbreviations

VDR: Vitamin D Receptor; TMA: Tissue Microarray; ER: oestrogen receptor; CK: Cytokeratin; EGFR: Epidermal Growth Factor Receptor; UDH: Usual Ductal Hyperplasia; ADH: Atypical Ductal Hyperplasia; CCL: Columnar Cell Lesions

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NL performed the practical work, analysed the data and drafted the manuscript. BS, DM and MG participated in the practical work. DV, LAV and FM analysed the data. JP and JLC designed the study and contributed to the manuscript. FS conceived the study, participated in its design and coordination, analysed the data and contributed to the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This study was supported by four research grants (Nair Lopes: SFRH/BD/39208/2007; Diana Martins: SFRH/BD/66152/2009; Madalena Gomes: PIC/IC/83264/2007; José Luis Costa: SFRH/BPD/20370/2004) and by a scientific project (PIC/IC/83264/2007), both financed by Fundação para a Ciência e Tecnologia (Portugal). Salary support was provided for Joana Paredes from Programa Ciência 2007, also financed by Fundação para a Ciência e Tecnologia (Portugal).

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Received: 20 April 2010 Accepted: 11 September 2010
Published: 11 September 2010

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Pre-publication history
The pre-publication history for this paper can be accessed here:
http://www.biomedcentral.com/1471-2407/10/483/prepub

doi:10.1186/1471-2407-10-483
Cite this article as: Lopes *et al.*: Alterations in Vitamin D signalling and metabolic pathways in breast cancer progression: a study of VDR, CYP27B1 and CYP24A1 expression in benign and malignant breast lesions Vitamin D pathways unbalanced in breast lesions. *BMC Cancer* 2010 **10**:483.

PUBLICATIONS

Paper 8

Human Pathology (2011) **xx**, xxx–xxx

Human PATHOLOGY

www.elsevier.com/locate/humpath

Original contribution

Molecular phenotypes of matched in situ and invasive components of breast carcinomas☆

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Received 14 May 2010; revised 26 July 2010; accepted 3 August 2010

Keywords:
In situ breast carcinoma;
Invasive breast carcinoma;
Molecular profile;
Tissue microarray

Summary The current system of pathologic classification of human breast cancers does not take into account the biologic determinants of prognosis, nor is there a consensus regarding the progression from in situ to invasive carcinoma. The present study compared the molecular phenotypes of in situ and invasive components of breast cancer in the same sample. We built a series of 189 in situ and invasive carcinomas using tissue microarrays and classified them according to their immunoprofiles regarding estrogen receptor, progesterone receptor, human epidermal growth factor receptor 2, epidermal growth factor receptor, cytokeratin 5, P-cadherin, and the antigen Ki-67 into luminal A and B, human epidermal growth factor receptor 2 overexpressing, and basal-like carcinomas. We also correlated the subgroups of carcinomas with some of the classical prognostic factors such as histologic grade, tumor size, and lymph node metastasis, as well as with the age of the patient at diagnosis. The overall concordance on the molecular phenotypes between in situ and invasive components was 94%. For the in situ component, 63% of the cases were luminal A; 15%, luminal B; 12%, human epidermal growth factor receptor 2 overexpressing; and 7%, basal-like. Regarding the invasive component, 61% of the cases were luminal A; 16%, luminal B; 12%, human epidermal growth factor receptor 2 overexpressing; and 8%, basal-like. The present study allowed the identification of different immunoprofiles of in situ and invasive breast carcinomas using a specific panel of biomarkers and showed that in most cases, there is a concordance between in situ and invasive component profiles, supporting the theory of parallel disease in breast tumorigenesis.

☆ Funding: This work was partially supported by research grants from Diana Martins: FCT- SFRH/BD/66152/2009; Bárbara Sousa (collaboration project at IPATIMUP); Nair Lopes: FCT-SFRH/BD/39208/2007; Madalena Gomes: PIC/IC/83264/2007; André Albergaria: FCT-SFRH/BD/15316/2005; and Joana Paredes: Ciência 2007—Portuguese Foundation for Science and Technology (FCT). IPATIMUP is an Associate Laboratory of the Portuguese Ministry of Science, Technology and Higher Education and is partially supported by FCT, the Portuguese Foundation for Science and Technology.

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doi:10.1016/j.humpath.2010.08.024

1. Introduction

Breast cancer is the most common cancer in women, with more than 1 million cases occurring worldwide annually [1]. Despite significant diagnostic and therapeutic innovations, the effect on the mortality rate has been modest. One of the factors contributing to this limited success is the relative lack of understanding of the natural history of this disease [2]. For example, the transition from in situ to invasive carcinoma is still a poorly understood event [3].

Nowadays, it is widely stated that the natural history of breast cancer involves progression through clinical and pathologic stages [3,4] from premalignant hyperplastic breast lesions, with or without atypia, to carcinoma in situ and then invasive carcinoma [5-7]. On the basis of molecular, epidemiologic, and pathologic studies, ductal carcinoma in situ (DCIS) is thought to be a precursor of invasive ductal carcinoma [4]. Although this model is supported by clinical and molecular research [8-11], it is only a starting point to understand breast tumorigenesis, as the relation between preinvasive lesions and invasive carcinoma remains unclear [12]. From the available data, 2 models have been proposed recently to explain the transition from DCIS to invasive breast carcinoma (IBC). The first one, the *theory of linear progression* [5,7,13], suggests that low-grade DCIS progresses to high-grade DCIS and then to invasive ductal breast carcinoma. This model implies that tumor progression follows a linear pattern. However, there is evidence that some in situ carcinomas never progress to invasion and that some DCIS have more genetic alterations than some invasive carcinomas [14], a finding which does not fit in this multistep model. Consequently, a second model of breast cancer tumorigenesis has been proposed: the *theory of the parallel disease*, wherein low-grade DCIS tends to progress to low-grade invasive ductal breast cancer, whereas high-grade DCIS tends to progress to high-grade invasive breast cancer [12]. In this model, a specific subtype of DCIS matches a specific subtype of invasive breast cancer.

Gene expression profiling is known to be a powerful tool for identifying tumor molecular profiles and for correlating gene expression profiles with outcome in breast cancer [14]. In addition, it has been an important tool to explore the transcriptional program that leads to invasion, comparing in situ and invasive carcinomas. Recently, Dalgin et al [15] studied 36 breast cancer patients with different pathologic stages of disease and revealed a hierarchical portrait of breast cancer progression, identifying genes and pathways for each stage, grade, and molecular subtype. These authors suggested that the heterogeneity of the disease across molecular subtypes is higher than the heterogeneity of disease progression within a subtype, suggesting that tumors with different molecular profiles are in fact distinct diseases.

Several studies have concentrated on the identification of specific biomarkers that could define the subtypes of in situ and IBCs [16-18]. Our group and others demonstrated that it is possible to translate the molecular classification, using immunohistochemistry (IHC) and tissue microarrays (TMAs) [18], where estrogen and progesterone receptors (ER and PgR) and human epidermal growth factor receptor 2 (HER-2) expression identify luminal A and B and HER-2 overexpression subtypes, whereas tumor protein 63 (p63), cytokeratin 5 (CK5), and P-cadherin (P-cad) allow the identification of basal-like tumors [17]. Recently, Paredes et al [18] also demonstrated the importance of P-cad and CK5 as useful adjunct markers to distinguish the basal-like subtype among the in situ carcinomas.

However, it was never determined whether the in situ and invasive carcinomas that develop in a particular breast cancer patient belong to the same molecular subtype or are different entities belonging to different molecular profiles.

In this study, our aim was to compare the molecular phenotypes of in situ and invasive components of breast cancer in the same sample, using IHC and TMAs and a specific panel of biomarkers, previously described by our group [17,18].

2. Materials and methods

2.1. Tumor specimens

One hundred eighty-nine formalin-fixed, paraffin-embedded samples harboring in situ and IBCs in the same block were collected from the archives of the Pathology Institute of Araçatuba, São Paulo, Brazil (1996-2006). All cases were classified from hematoxylin and eosin (H&E)–stained sections. The DCIS samples were subdivided into 3 groups: low, intermediate, and high grade, according to the nuclear grade and the extent of necrosis, as previously published by our group [19]. Briefly, tumors harboring nuclear grade 3 were all considered high grade, whereas tumors with nuclear grade 1 or 2 with necrosis were considered intermediate grade, and those of nuclear grades 1 and 2 without necrosis were considered low grade. Invasive breast cancers were classified as grade I, II, or III according to the method described by Elston and Ellis [20]. Some relevant data were available for analysis, including age and clinicopathologic features such as tumor size and lymph node metastasis.

2.2. TMAs construction

Representative areas of the in situ and IBCs were selected on H&E-stained sections and marked on the corresponding paraffin blocks. Two 2-mm tissue cores were obtained from each selected specimen (donor block) and deposited in a paraffin (receptor) block using a TMA workstation (TMA Builder ab1802; Abcam, Cambridge, UK). Twenty-two TMA blocks were constructed, each containing 24 tissue cores (4 × 6). In each TMA block, nonneoplastic breast and liver tissue cores were included as a control and a TMA guide, respectively. After the construction, 2-μm tissue sections were cut and attached to Superfrost Plus glass slides.

An H&E-stained section from each TMA block was reviewed to confirm the presence of morphologically representative areas of the original lesions.

2.3. Immunohistochemistry

The sections were immunostained with primary monoclonal antibodies against ER, PgR, HER-2, epidermal growth factor receptor (EGFR), CK5, P-cad, and Ki-67. Immunostaining for ER, HER-2, and CK5 was performed using the streptavidin-biotin peroxidase technique (LabVision, Fremont, CA), whereas for PgR, EGFR, P-cad, and Ki-67, a horseradish peroxidase–labeled polymer (DakoCytomation, Carpinteria, CA) was used.

Antigen unmasking for ER, PgR, HER-2, and Ki-67 was carried out using 1:100 commercial citrate buffer, pH 6.0 (Vector Laboratories, Burlingame, CA) at 98°C, whereas a dilution of 1:10 from *tris*-ethylenediaminetetraacetic solution at pH 9.0 (DakoCytomation) was used for CK5 and P-cad. Epitope retrieval for EGFR was performed by proteolytic digestion (pepsin A, 4 g/L; Sigma-Aldrich, St. Louis, MO, USA) at 37°C.

The antigen retrieval time, antibodies, dilutions, and suppliers are listed in Table 1. After the antigen retrieval procedure, the slides were washed in phosphate-buffered saline and submitted to blockage of endogenous peroxidase activity by incubation of the slides in 3% hydrogen peroxide (Panreac, Barcelona, Spain) in methanol (Sigma-Aldrich). The slides were further incubated with blocking serum (LabVision Corporation kit) for 15 minutes and then incubated with the primary antibodies. After washes, the slides were incubated with biotinylated secondary antibody, followed by streptavidin-conjugated peroxidase (LabVision). Diaminobenzidine was used as a chromogen (DakoCytomation).

Table 1 Sources and dilutions of primary antibodies used in immunohistochemistry staining

Antibody	Clone	Manufacturer	Time of incubation (min)	Dilution	Antigen retrieval (min)
ER	SP1	Neomarkers (Fremont, CA, USA)	60	1:100	30
PgR	1A6	Novocastra (Newcastle, UK)	60	1:40	30
HER-2	SP3	Neomarkers	30	1:80	30
P-cad	56	Transduction Labs (Franklin Lakes, NJ, USA)	60	1:50	30
CK5	XM26	Neomarkers	60	1:50	30
EGFR	31G7	Zymed	60	1:100	30
Ki67	SP6	Neomarkers	60	1:300	30

For PgR, EGFR, P-cad, and Ki-67 staining, the secondary antibody was associated with horseradish peroxidase–labeled polymer (DakoCytomation) and immediately revealed with diaminobenzidine. Tissues were then counterstained with Mayer hematoxylin, dehydrated, and covered using a permanent mounting solution (Zymed, San Francisco, CA).

Positive controls were included in each run to guarantee the reliability of the assays. Nonneoplastic breast tissues, as well as normal breast surrounding the neoplastic cells, were considered internal controls.

2.4. Quantification of immunostaining

The IHC results were evaluated by 2 pathologists (F.S., F. M.). Both ER and PgR were examined for staining intensity, ranked from 1 to 3 (1, weak; 2, moderate; 3, strong) and extent, ranked from 1 to 10 (1, 0-10% cells; 2, 11%-20% cells; 3, 21%-30% cells; 4, 31%-40% cells; 5, 41%-50% cells; 6, 51%-60% cells; 7, 61%-70% cells; 8, 71%-80% cells; 9, 81%–90% cells; 10, 91%-100% cells) using the H-score method, which is used for other nuclear receptors as well [21,22]. The scores for intensity and extension were multiplied, and the cases were considered negative when the score was less than 4 and positive from 5 to 30. Concerning Ki-67, tumors with unequivocal nuclear staining in more than 14% of the cells were classified as highly proliferative, whereas tumors with less than 14% positive cells were considered to show low proliferation [23]. We considered positive the cases with membranous staining for P-cad and cytoplasmic staining for CK5 in at least 10% of the neoplastic cells. Expression of HER-2 was evaluated according to the DakoCytomation Hercept Test scoring system [24]. Cases were considered positive (overexpression) when immunostaining was classified as 3+. If a case is classified as 2+ by IHC, fluorescence in situ hybridization analysis was performed to determine if the tumor had *HER2* amplification. If amplification was confirmed, the tumor was classified as positive. If the tumor did not demonstrate amplification, it was considered negative. Staining for EGFR was also classified according to the Hercept Test scoring system. However, breast carcinomas were considered positive whenever the immunostaining was 2+ or 3+. Cases that were ER+ or PgR+ and HER-2 negative were classified as luminal A; cases ER+/PgR+ and HER-2+ or ER+ and with a high proliferative index (Ki-67+) were considered luminal B; ER− and PgR− and HER-2+ cases were classified as HER-2-overexpressing; cases that were negative for ER, PgR, and HER-2 and positive for EGFR or CK5 or P-cad were considered basal-like. Cases that lacked expression of all tested markers were considered "unclassified."

2.5. Statistical analysis

StatView 5.0 (SAS Institute Inc, Cary, NC) was used for statistical analysis. Univariate associations between ER, PgR, HER-2, EGFR, CK5, P-cad, KI67, tumor size,

Table 2 Frequencies of immunohistochemically defined subtypes of in situ and invasive breast cancers

Subtype	Frequency in situ component, n (%)	Frequency invasive component, n (%)
Luminal A	120/189 (63)	116/189 (61)
Luminal B	28/189 (15)	31/189 (16)
HER-2 overexpressing	23/189 (12)	23/189 (12)
Basal-like	13/189 (7)	14/189 (8)
Unclassified	5/189 (3)	5/189 (3)

histologic grade, and lymph node metastases in the presence of DCIS and invasive breast cancer were assessed using contingency tables and χ^2 tests. In all statistical analyses, $P \leq .05$ was considered significant.

3. Results

We performed IHC on each set of the 22 TMA slides for ER, PgR, HER-2, P-cad, CK5, EGFR, and Ki-67. Tables 2 and 3 summarize the clustering of a total of 189 immunohistochemically interpretable cases to allow sample characterization into 1 of the 5 previously described molecular subtypes. The molecular classification was made in an individual way for each of the tumor components (in situ and invasive) in the same block.

3.1. Evaluation of the in situ component

Among the in situ components, we observed that 63% of all tumors were considered luminal A, whereas the luminal B and HER-2–overexpressing subtypes comprised 15% and 12% of the cases, respectively. Basal-like tumors represented 7%, and the ones with null phenotype/unclassified were 3% (Table 2).

Because luminal cancer subtypes (A and B) were defined as positive for hormone receptors (ER, PgR), the percentage of cases positive for these 2 immunohistochemical markers was extremely high, as expected, with a higher prevalence for ER positivity (Table 3). For the luminal A subtype, 95% and 66% of the cases were ER and PgR positive, respectively, whereas for luminal B, 100% were positive for ER and 61% were positive for PgR. As initially defined, all luminal A tumors were negative for HER-2, and all luminal B lesions were positive for this marker. In the specimens negative for hormone receptors, all the cases overexpressing HER-2 were included in the HER-2–overexpressing cancer subtype, which are being the triple-negative ones (negative for ER, PgR, and HER-2) divided into basal-like or unclassified, according to the positivity for P-cad, CK5, and EGFR. Among the basal-like tumors, P-cad was the most prevalent marker, with 92% of the cases being positive, whereas only 23% and 38% of the cases were positive for EGFR and CK5, respectively.

Although basal markers are most commonly expressed in basal-like tumors, these can also be present in other cancer subtypes, if at a lower frequency. Concerning EGFR, although there were almost no positive cases in the luminal A and B subtypes, 10% of HER-2–overexpressing tumors also expressed EGFR. Also, CK5 was expressed by 17% of the HER-2–overexpressing in situ carcinomas, whereas only 3% and 7% of the tumors classified as luminal A or B, respectively, showed CK5 expression. Expression of P-cad also was common in HER-2–overexpressing tumors, which is being positive in almost half the cases (48%). Concerning the luminal cancer subtypes, P-cad expression was more abundant in luminal B (14%) than in luminal A (8%) lesions.

Concerning cell proliferation indexes, addressed by Ki-67 staining, basal-like tumors were the ones showing higher values (28%), followed by luminal B (19%). When we studied the association between the in situ histologic grade and molecular cancer subtypes (Fig. 1), we found that luminal A tumors were frequently classified as low grade (49%),

Table 3 Comparison of molecular subtypes and biomarkers for in situ and invasive components

		Luminal A		Luminal B		HER-2 overexpressing		Basal-like		*P*	
		In situ	Invasive	In situ	Invasive	In situ	Invasive	In situ	Invasive	In situ	Invasive
ER	+	114 (95%)	108 (93%)	28 (100%)	31 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	$P \leq .0001$	$P \leq .0001$
	−	6 (5%)	8 (7%)	0 (0%)	0 (0%)	23 (100%)	23 (100%)	13 (100%)	14 (100%)		
PR	+	79 (66%)	79 (68%)	17 (61%)	13(42%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	$P = .0002$	$P = .0001$
	−	41 (34%)	37 (32%)	11 (39%)	18 (58%)	23 (100%)	23 (100%)	13 (100%)	14 (100%)		
HER-2	+	0 (0%)	0 (0%)	15 (54%)	14 (45%)	23 (100%)	23 (100%)	0 (0%)	0 (0%)	$P \leq .0001$	$P \leq .0001$
	−	120 (100%)	116 (100%)	13 (46%)	17 (55%)	0 (0%)	0 (0%)	13 (100%)	14 (100%)		
EGFR	+	1 (1%)	1 (1%)	0 (0%)	0 (0%)	2 (10%)	2 (9%)	3 (23%)	3 (21%)	$P = .004$	$P = .001$
	−	119 (99%)	115 (99%)	28 (100%)	31 (100%)	21 (90%)	21 (91%)	10 (77%)	11 (79%)		
CK5	+	4 (3%)	2 (2%)	2 (7%)	0 (0%)	4 (17%)	5 (22%)	5 (38%)	5 (36%)	$P = .001$	$P \leq .0001$
	−	116 (97%)	114 (98%)	26 (93%)	31 (100%)	19 (83%)	18 (78%)	8 (62%)	9 (64%)		
P-cad	+	9 (7%)	9 (8%)	4 (14%)	4 (13%)	11 (48%)	11 (48%)	12 (92%)	13 (93%)	$P \leq .0001$	$P \leq .0001$
	−	111 (93%)	107 (92%)	24 (86%)	27 (87%)	12 (52%)	12 (52%)	1 (8%)	1 (7%)		

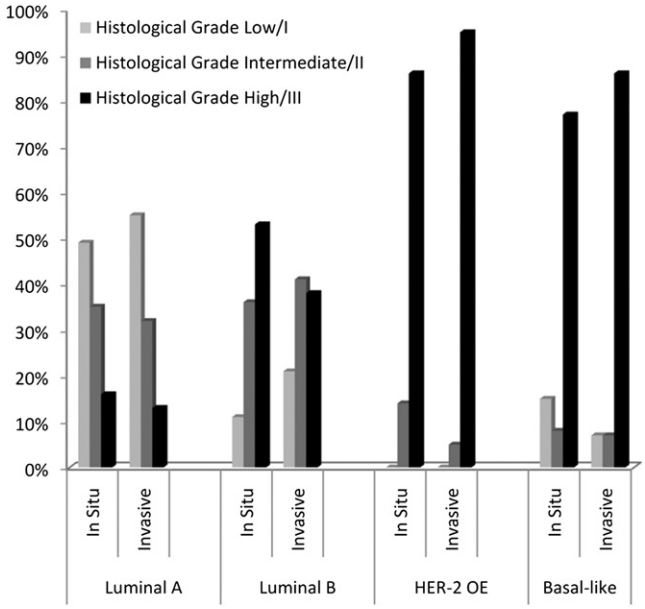

Fig. 1 Comparison of histologic grade among molecular subtypes (luminal A, luminal B, HER-2, and basal) in situ and invasive components. All correlations were statistically significant ($P \leq .05$).

whereas most luminal B carcinomas were classified as intermediate grade (53%); HER-2–overexpressing and basal-like cases were more often of high grade (86% and 77%).

3.2. Evaluation of the invasive component

For the invasive component (Table 2), the luminal A subtype represented 61% of all the tumors. Luminal B and HER-2–overexpressing invasive tumors corresponded to 16% and 12%, respectively, whereas basal-like tumors comprised 8% of the cases. Only 3% of the invasive carcinomas were null phenotype/unclassified. For the luminal A cancer subtype, 93% and 68% of the cases were ER and PgR positive, respectively, whereas for luminal B, 100% were positive for ER and 42% were positive for PgR. Again, all luminal A tumors were negative for HER-2, as expected, and 45% of luminal B cases were positive for this marker. The invasive carcinomas overexpressing HER-2 and negative for hormone receptors were included in the HER-2–overexpressing cancer subtype. In triple-negative basal-like invasive tumors, as described for the in situ component, P-cad expression was the most prevalent basal marker, with 93% positive cases, whereas only 21% and 36% of the cases were positive for EGFR and CK5, respectively (Table 3).

When we studied the expression of basal markers in cancer subtypes other than the basal-like, we found results similar to the ones described for the in situ component of this breast cancer series. Concerning EGFR, exactly the same frequencies were found: 1% and 0 of the cases expressed this receptor in luminal A and B subtypes, respectively, whereas 9% of HER-2–overexpressing tumors coexpressed these 2 tyrosine kinase receptors. CK5 was expressed by 22% of the HER-2–overexpressing invasive carcinomas, whereas only 2% or none of the tumors classified as luminal A or B, respectively, showed CK5 expression. Again, P-cad expression was highly expressed in HER-2–overexpressing tumors (48%), but it was expressed by only 8% of luminal A and 13% of luminal B IBCs.

For Ki-67, the tumors included in the basal-like and luminal B subtypes had the highest proliferative indexes (29% and 25%, respectively). Regarding the histologic grade (Fig. 1), we found that luminal A invasive tumors were often grade I (55%), whereas luminal B lesions were from intermediate (41%) to high grade (38%); once more, HER-2–overexpressing and basal-like tumors were more regularly classified as grade III (95% and 86%, respectively).

3.3. Combined evaluation of the in situ and invasive counterparts in the same patient

Most cases (93%) maintained the molecular classification when the in situ and invasive components were compared (Fig. 2); there were just 13 cases (7%) in which the 2 areas were classified differently (Table 4). One of the cases was unclassified for the in situ component (negative for all markers), but basal-like in the invasive counterpart in which P-cad expression was seen. Both components were high grade. Another case was classified as an in situ luminal A carcinoma but was unclassified in the invasive component because of the absence of expression of both hormone receptors (Fig. 2D). Interestingly, although both components were intermediate grade, the invasive counterpart had a higher proliferative index. Four cases were classified as luminal B for the in situ component but luminal A for invasive counterpart because of the low proliferative index, with the exception of one case that lacked HER-2 expression also in the invasive area. Interestingly, in this case, the loss of expression was accompanied by a difference in the histologic grade: high grade in the in situ portion and grade II in the invasive counterpart.

Finally, 7 cases that were classified as in situ luminal A carcinomas were classified as luminal B in the invasive portion because of a higher proliferative index. Other than the increase in cell proliferation, no alterations were noticed in histologic grade.

In general terms, we can conclude that there are no important modifications of the breast cancer molecular classification in most cases when the transition from an in situ to an invasive carcinoma occurs. However, when we compared the expression of the different biomarkers individually (Table 3), we could find subtle differences between components, which can add some biologic information to the in situ/invasive transition. In the luminal A cases, in addition to the higher proliferative rate in the invasive component in 7 cases, just 3 cases showed P-cad expression in the invasive component. No alterations in hormone receptors were found between in situ and invasive transition. In luminal B tumors, no alterations were found for

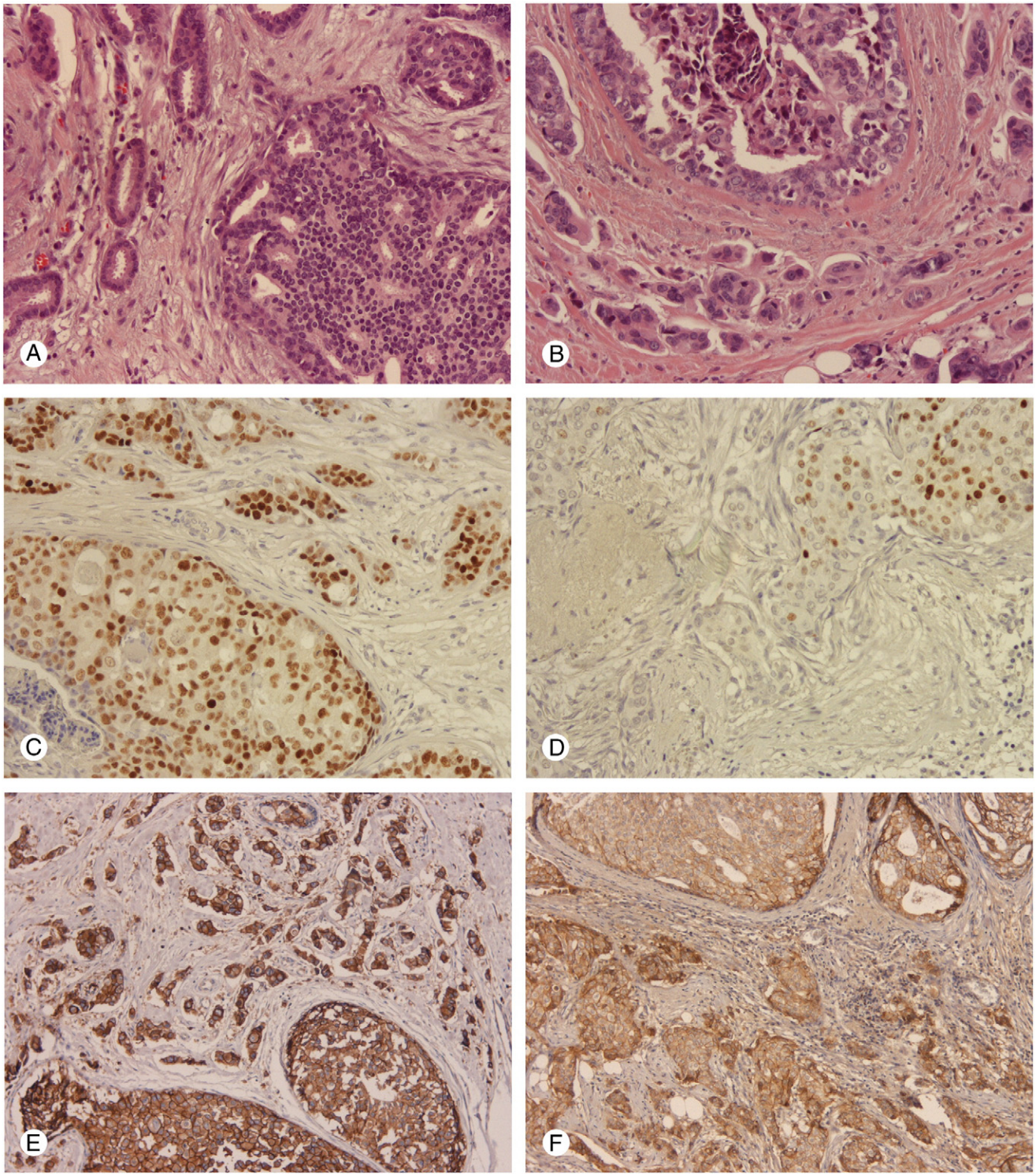

Fig. 2 Expression of proteins studied by IHC staining on TMAs for in situ and invasive components. A and B, H&E staining of low-/I and high-grade/III histologic grade, respectively. C, ER expression. D, Loss of ER expression in invasive component. E, HER-2 staining. F, P-cad staining. Original magnifications: A-D ×200; E, F ×100.

ER expression when the in situ and invasive components were compared. However, there were 4 cases that lost PgR expression in the invasive carcinoma, with the transition from a high-grade in situ carcinoma to a grade II invasive tumor in 2 cases; in the remaining 2, there were no alterations in grade. The other differences were in basal markers, such as CK5 and P-cad, with a loss of 7% and 3% of expression from in situ to invasive tumors, respectively. Regarding the HER-2–overexpression lesions, 7 did not show expression of any basal marker, whereas 14 cases showed concomitant

Table 4 Discordant molecular classifications between in situ and invasive components

No.	In situ component	Invasive component
7	Luminal A	Luminal B
4	Luminal B	Luminal A
1	Luminal A	Unclassified
1	Unclassified	"Basal-like"

expression of EGFR, CK5, or P-cad together with HER-2. Of these, P-cad was the most prevalent. There were 3 cases that gained expression of basal markers in the transition from in situ to invasive carcinoma, namely, 2 with CK5 and 1 with P-cad. Only this last case changed histologic grade (from an intermediate in situ grade to a grade III invasive tumor). In the basal-like subtype, most cases were P-cad positive in both components (11 cases). However, there was 1 case that lost P-cad in the invasive fraction, but because it expressed CK5 in the invasive component, its molecular classification did not change.

Molecular subtypes of in situ and invasive breast cancers did not differ with the histologic grade ($P < .0001$ and $P = .0002$ for in situ and invasive counterparts, respectively). High-grade lesions were associated with the HER-2–overexpressing and basal-like phenotypes in both the in situ and invasive components. Low-grade lesions were frequently of the luminal A phenotype. In the luminal B phenotype, the in situ component was more frequently high grade (53%), whereas the invasive counterpart was intermediate grade (41%).

As mentioned above, the only cases with alterations in molecular classification were not accompanied by differences in histologic grade. However, there were some alterations in histologic grade in individual cases: 9 lesions with intermediate in situ components were grade III in the invasive counterpart, whereas 5 classified as in situ low grade were grade II lesions when we analyzed the invasive component. In cases where there was a decrease in the histologic grade, 12 cases classified as high grade in the in situ component were grade II in the invasive counterpart; 1 case was high grade in the in situ component and grade I in the invasive one, and 10 cases classified as intermediate grade in the in situ component were grade I in the invasive area.

4. Discussion

Two main branches of breast tumorigenesis have been distinguished: one supports the multistep model and the other the theory of parallel disease, where a specific subtype of DCIS matches a specific subtype of invasive breast cancer [12]. In 1997, Grupta et al [25], studying 300 patients with IBC associated with DCIS, demonstrated that the degree of differentiation of DCIS correlated with the grade of the invasive carcinoma and the clinical outcome. They also showed that patients with invasive breast cancer displayed the same mutations as patients with preinvasive and invasive lesions. In fact, recent data [26] demonstrate that the most dramatic alterations in gene expression patterns occur during the transition from normal breast tissue to DCIS [27,28], not from in situ to invasive. In contrast, Tamimi et al [29], studying 272 DCIS and 2249 invasive independent tumors, showed that in situ and invasive phenotypes had different prevalences. These authors found a higher prevalence of luminal B and HER-2–overexpressing profiles among DCIS tumors. However, analyzing independent series of in situ and invasive tumors [17,18], no differences were found in molecular subtype prevalence. So probably, the higher percentage of the HER-2 phenotype in DCIS in the series described by Tamimi et al [29] was attributable to the examination of a mammographically screened population and does not reflect a basis of progression to invasive tumors.

The great advantage of our series of 189 breast carcinomas, which was characterized by several immunohistochemical markers, relies on the existence of in situ and invasive components in the same sample. We classified the in situ and invasive tumors into 4 main molecular subtypes (luminal A, luminal B, HER-2–overexpressing, and basal-like). When the components were compared, we verified that there were no significant differences in the molecular classification of in situ and invasive tumors, which led us to conclude that different molecular subtypes have different progression forms, low-grade in situ tumors evolving into low-grade invasive tumors and high-grade in situ tumors into high-grade invasive tumors [15].

Differences in molecular profiles between the in situ and invasive carcinoma areas were observed in only 13 cases, which seems more likely to be attributable to technical immunohistochemical issues than a reflection of changes in tumor biology. One case classified as luminal A in the in situ component lost ER expression and became unclassified in the invasive component. Another case, which did not express any of the markers used for classification (unclassified) in the in situ component, gained expression of P-cad in the invasive component and could be characterized as having a basal-like phenotype. In the remaining 11 cases, the changes were from luminal A to B or vice versa, and these alterations can be attributed to the fact that the criteria for classification of the luminal B subtype are not well established. Although some luminal B tumors can be identified by their expression of HER-2, the chief biologic distinction between luminal A and B is the proliferative signature, including genes such as Ki-67. Chang and collaborators [23], using 14% as a cutoff, supported Ki-67 as a well-established cell proliferation marker in cancer and emphasized its role as a biomarker candidate for identification of luminal B tumors. We also used this cutoff, which allowed us to distinguish some luminal B tumors that the standard biomarker panel (ER, PgR, and HER-2) did not identify. Interestingly, and although the percentages are close to that of the luminal B subtype, the basal-like tumors had higher proliferative

indices than the other subtypes in both the in situ and invasive components (28% and 29%, respectively). Among these fractions, the invasive one had a higher proliferative rate, which can be associated with an increase in cell proliferation when invasion occurs, and with the poor prognosis associated with this molecular subtype.

An association between histologic grade and molecular phenotype has been demonstrated, with low-grade invasive tumors usually having the luminal A phenotype, whereas high-grade tumors are more prevalent among HER-2–overexpressing and basal-like subtypes [29,30]. Moreover, the HER-2–overexpressing and basal-like subtypes are associated with a poor prognosis. In our series, in in situ breast cancers, HER-2 and basal-like subtypes were more frequently high grade than low or intermediate grade (86% and 77%, respectively). These results were consistent for invasive tumors, because 95% of HER-2+ and 86% of basal-like tumors had high histologic grades. It was interesting to note the percentages of the luminal B type among DCIS and invasive components, where intermediate/II and high grade prevail in both, with 36% and 54% for the in situ component, as well as 41% and 38% for the invasive counterpart, respectively. This similarity between intermediate/grade II and high grade probably is secondary to the cutoff used, which enriched our series in luminal B cases.

We also looked for cases that showed alterations simultaneously in biomarker expression and histologic grade and found 8 cases. It is important to say that these alterations were not accompanied by alterations in the molecular classification. Two cases graded as having an intermediate in situ component were grade III in the invasive component, accompanied by gain of P-cad in one case and its loss in the other. Other 2 cases, with the concordant loss of PgR expression, classified as high grade in the in situ component, were grade II in the invasive counterpart. One case also lost PgR expression but changed from intermediate in situ to grade I in the invasive counterpart. It is also interesting that we had 2 cases that lost PgR expression and 1 basal marker, P-cad or CK5, simultaneously and were classified as high-grade in situ and grade II in the invasive component. Finally, the remaining case lost CK5 and changed from intermediate to grade I.

We have shown that the prevalence of molecularly defined phenotypes did not differ significantly between DCIS and invasive breast cancers; probably, the molecular alterations that drive invasion occur before the morphologic modification of the lesion [31,32]. Dalgin et al [15] also confirmed that the cancer phenotype develops early (in the early hyperplasia or DCIS stage), and each subtype progresses along its own specific pathway, as if each was a distinct disease.

In conclusion, with this work, we showed that it is possible to identify different immunohistochemical profiles of in situ and invasive breast cancer using a small panel of biomarkers (ER, PgR, HER-2, EGFR, CK5, P-cad, and Ki-67) and that the technique of TMA is useful, efficient, and reliable in the characterization and subclassification of a large number of cases. Concerning the comparison of in situ and invasive components, we found that in 176 (93%) of the 189 cases, the molecular classification was identical in the 2 components, which supports the theory of parallel disease; that is, that in the progression of most breast cancer cases, there is a commitment of the in situ subtype carcinoma to a specific subtype of invasive carcinoma. Otherwise, the finding supports the view that the molecular phenotype is established at the DCIS stage. Although there has been an improvement in understanding the pathways of breast tumorigenesis, little is known about the mechanisms associated with the transition from in situ to invasive carcinomas. More than just genetic alterations in the tumor cells, the codependency of epithelial cells and stroma can regulate tumor progression. Recently, it was demonstrated that myoepithelial cells can have a particular role in tumor invasion. Studying normal myoepithelial cells and the ones associated with DCIS, Schnitt [32,33] demonstrated that the last ones differ substantially from the normal, showing down-regulation of genes involved in the normal function of cells and up-regulation of genes associated with invasion. There is an immediate need to characterize new molecules that not only uncover the molecular biology of in situ carcinoma and its transition to invasive breast cancer, but also the transcriptional program that drives the invasive growth of each molecular subtype.

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PUBLICATIONS

Paper 9

Histol Histopathol (2011) 26: 1279-1286

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

GLUT1 and CAIX expression profiles in breast cancer correlate with adverse prognostic factors and MCT1 overexpression

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Summary. The goal of the present work was to evaluate the correlation of glucose transporter 1 (GLUT1) and carbonic anhydrase IX (CAIX) with the monocarboxylate transporters 1 (MCT1) and 4 (MCT4) and their chaperone, CD147, in breast cancer. The clinico-pathological value of GLUT1 and CAIX was also evaluated. For that, we analysed the immunohistochemical expression of GLUT1 and CAIX, in a large series of invasive breast carcinoma samples (n=124), previously characterized for MCT1, MCT4 and CD147 expression. GLUT1 expression was found in 46% of the cases (57/124), while CAIX was found in 18% of the cases (22/122). Importantly, both MCT1 and CD147, but not MCT4, were associated with GLUT1 and CAIX expression. Also, GLUT1 and CAIX correlated with each other. Concerning the clinico-pathological values, GLUT1 was associated with high grade tumours, basal-like subtype, absence of progesterone receptor, presence of vimentin and high proliferative index as measured by Ki-67. Additionally, CAIX was associated with large tumour size, high histological grade, basal-like subtype, absence of estrogen and progesterone receptors and presence of basal cytokeratins and vimentin expression. Finally, patients with CAIX positive tumours had a significantly shorter disease-free survival.

The association between MCT1 and both GLUT1 and CAIX may result from hypoxia-mediated metabolic adaptations, which confer a glycolytic, acid-resistant and more aggressive phenotype to cancer cells.

Key words: GLUT1, CAIX, Monocarboxylate transporters (MCTs), CD147/EMMPRIN, Breast carcinoma, Immunohistochemistry

Introduction

Early epithelial carcinogenesis occurs under hypoxic conditions, since altered cells are separated from the vascularised stroma, source of oxygen and nutrients. To maintain the needed ATP levels, cancer cells increase their rates of glycolysis, acquiring a significant proliferative advantage. However, this phenotype leads to an overload of lactic acid, which must be exported from the cell, causing a decrease in the extracellular pH.

Constitutive upregulation of glycolysis requires additional adaptations, namely, resistance to apoptosis and upregulation of membrane transporters to maintain normal intracellular pH (Gatenby and Gilles, 2004). The need to increase glucose uptake, to allow high glucose consumption rates, is achieved by upregulation of glucose transporters (GLUT) in the plasma membrane of cancer cells, especially the hypoxia-responsive GLUT1 (Ganapathy et al., 2009). However, the role of GLUT1 in breast cancer remains poorly elucidated (Younes et al., 1995; Kang et al., 2002).

Besides being an adaptation to high glycolytic phenotype, the acidic environment represents, *per se,* a

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Abbreviations: CA (carbonic anhydrase); ER (estrogen receptor); GLUT (glucose transporter); HIF-1α (hypoxia inducible-factor 1 alpha); MCT (monocarboxylate transporter); PR (progesterone receptor)

significant advantage for tumour cells since it is associated with increased migration, invasion and metastases, among others (Gatenby and Gilles, 2004; Gatenby et al., 2006). Although lactate produced by glycolysis under hypoxic conditions is a significant contributor to acidic extracellular pH, there is also a substantial contribution from carbonic acid (Helmlinger et al., 2002). In this context, the hypoxia-responsive carbonic anhydrase isoforms, CAIX and CAXII, emerge as important contributors to the regulation of cancer cell intracellular pH (Swietach et al., 2007; Chiche et al., 2009), with CAIX, in particular, being associated with poor prognosis in breast cancer (Chia et al., 2001; Trastour et al., 2007; Hussain et al., 2007; Tan et al., 2009; Chen et al., 2010).

Another important group of proteins involved in intracellular pH regulation are monocarboxylate transporters (MCTs), which are also responsible for transmembrane transport of lactate (Izumi et al., 2003). By performing these two inter-dependent activities (lactate transport coupled with a proton), MCTs appear as strong potential targets for cancer therapy. Indeed, there is evidence for the up-regulation of MCTs in tumours, such as high grade glial neoplasms (Mathupala et al., 2004), colorectal (Koukourakis et al., 2006; Pinheiro et al., 2008a), cervical (Pinheiro et al., 2008b), and breast carcinomas (Pinheiro et al., 2010). Besides analysing MCT expression in tumours, our group also assessed for the first time the clinico-pathological value of their overexpression (Pinheiro et al., 2008a,b, 2009a, 2010). MCT expression appears to be influenced by altered physiological conditions; however, the underlying molecular events involved in MCT regulation are poorly understood. Recently, it was demonstrated that proper expression and activity of MCT1 and MCT4 requires co-expression of CD147, also known as EMMPRIN or Basigin (Kirk et al., 2000; Wilson et al., 2005). Based on this, we described the association between CD147 and both MCT1 and MCT4 in human cervical (Pinheiro et al., 2009b), gastric (Pinheiro et al., 2009a) and breast cancer (Pinheiro et al., 2010). Furthermore, the hypoxia inducible-factor 1 alpha (HIF-1α), which regulates many genes codifying proteins involved in the glycolytic pathway (like GLUT1) and pH regulation (like the Na^+/H^+ exchanger NHE1 and both CAIX and CAXII) (Vaupel and Harrison, 2004), also regulates MCT1 (Perez et al., 2010) and MCT4 (Ullah et al., 2006; Perez et al., 2010). However, there is some controversy around MCT1 regulation by hypoxia, with some studies reporting MCT1 repression by hypoxia (Ullah et al., 2006; Sonveaux et al., 2008).

One of the goals of the present study was to evaluate the association between the HIF-1α downstream targets GLUT1 and CAIX and both MCT1 and MCT4, as well as their chaperone, CD147, in invasive breast carcinomas. We also intended to strengthen the clinico-pathological value of GLUT1 and CAIX in breast cancer.

Materials and methods

Case selection

Case selection was based on availability of follow up information and amount of material, ensuring adequate numbers for statistical analysis. Thus, a series of 124 formalin-fixed paraffin embedded breast carcinoma tissues was retrieved from the files of the Department of Pathology, Hospital do Divino Espírito Santo, Azores, Portugal, and from the Federal University of Santa Catarina, Florianopolis-SC, Brazil. Haematoxylin/eosin stained sections of all cases were reviewed by three pathologists (R.D., D.V. and F.S.). Tumour samples were organized into 14 tissue microarrays (TMAs), with 20 tumour cores (2 mm diameter) each, also including several samples of normal breast tissue. Each case was represented in the TMA by at least two cores. Relevant clinico-pathological data included tumour size (TNM), molecular subtype, histological grade, estrogen receptor (ER) and progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor 1 (EGFR), basal cytokeratins (CK5 and CK14), vimentin and Ki67 expression status. Information on lymph-node metastasis, disease-free survival and overall survival was also available. These tumour samples were previously analysed by our group for MCT1, MCT4 and CD147 expressions (Pinheiro et al., 2010).

The molecular classification was done by translating the immunohistochemistry results for ER, PR, HER2, EGFR, CK5, CK14, vimentin and Ki-67. Tumours positive for ER and/or PR were classified as luminal. Cases positive for ER/PR and for HER2 and/or high Ki-67 index were subclassified as luminal B. Cases classified as HER2 overexpressing were characterized by HER2 overexpression and negativity for ER/PR, and cases defined as "basal-like" were negative for ER/PR and HER2 and positive for at least one of the "basal markers" tested.

Immunohistochemistry

GLUT1 and CAIX detection

Immunohistochemistry was performed based on the streptavidin–biotin–peroxidase complex principle (Ultravision Detection System Anti-polyvalent, HRP, Lab Vision Corporation, Fremont, CA), using rabbit polyclonal primary antibodies raised against GLUT1 (ab15309, AbCam, Cambridge, UK, diluted 1:500) and CAIX (ab15086, AbCam, Cambridge, UK, diluted 1:2000). Briefly, deparaffinized and rehydrated sections were immersed in citrate buffer (0.01M, pH 6.0) heated up to 98ºC, in a water bath, for 10 minutes (GLUT1) or 20 minutes (CAIX) and washed in PBS. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 minutes, followed by

washing with PBS. Tissue sections were incubated with blocking solution for 10 minutes and incubated at room temperature with the primary antibody for 2 hours. Sections were then sequentially washed in PBS and incubated with biotinylated goat anti-polyvalent antibody for 10 minutes, streptavidin peroxidase for 10 minutes, and developed with 3,3'-diamino-benzidine (DAB+ Substrate System, Dako, Carpinteria, CA) for 10 minutes. Negative controls were performed by using adequate serum controls for the primary antibodies (N1699, Dako, Carpinteria, CA) and skin and gastric mucosa were used as positive controls for GLUT1 and CAIX, respectively. Tissue sections were counterstained with haematoxylin and permanently mounted.

Immunohistochemical evaluation

As described for MCTs and CD147 (Pinheiro et al., 2010), GLUT1 and CAIX immunohistochemical reactions were scored semi-quantitatively for plasma membrane staining as follows: 0: 0% of immunoreactive cells; 1: <5% of immunoreactive cells; 2: 5-50% of immunoreactive cells; and 3: >50% of immunoreactive cells. Also, intensity of staining was scored semi-qualitatively as follows: 0: negative; 1: weak; 2: intermediate; and 3: strong. The final score was defined as the sum of both parameters (extension and intensity), and grouped as negative (score 0 and 2) and positive (score 3-6). Evaluation of GLUT1 and CAIX immunohistochemical reactions were performed blindly by two independent observers (F.S., J.P.). Discordant results between observers were discussed in a double-head microscope and a final score was agreed, while discordant results between different cores of the same tumour were handled by choosing the highest staining intensity and finding the mean value for immunoreactive cells.

Statistical analysis

Data were stored and analysed using the Statview statistical software (SAS Institute Inc., Cary, NC). All comparisons were examined for statistical significance using Pearson's chi-square (χ^2) test or Fisher's exact

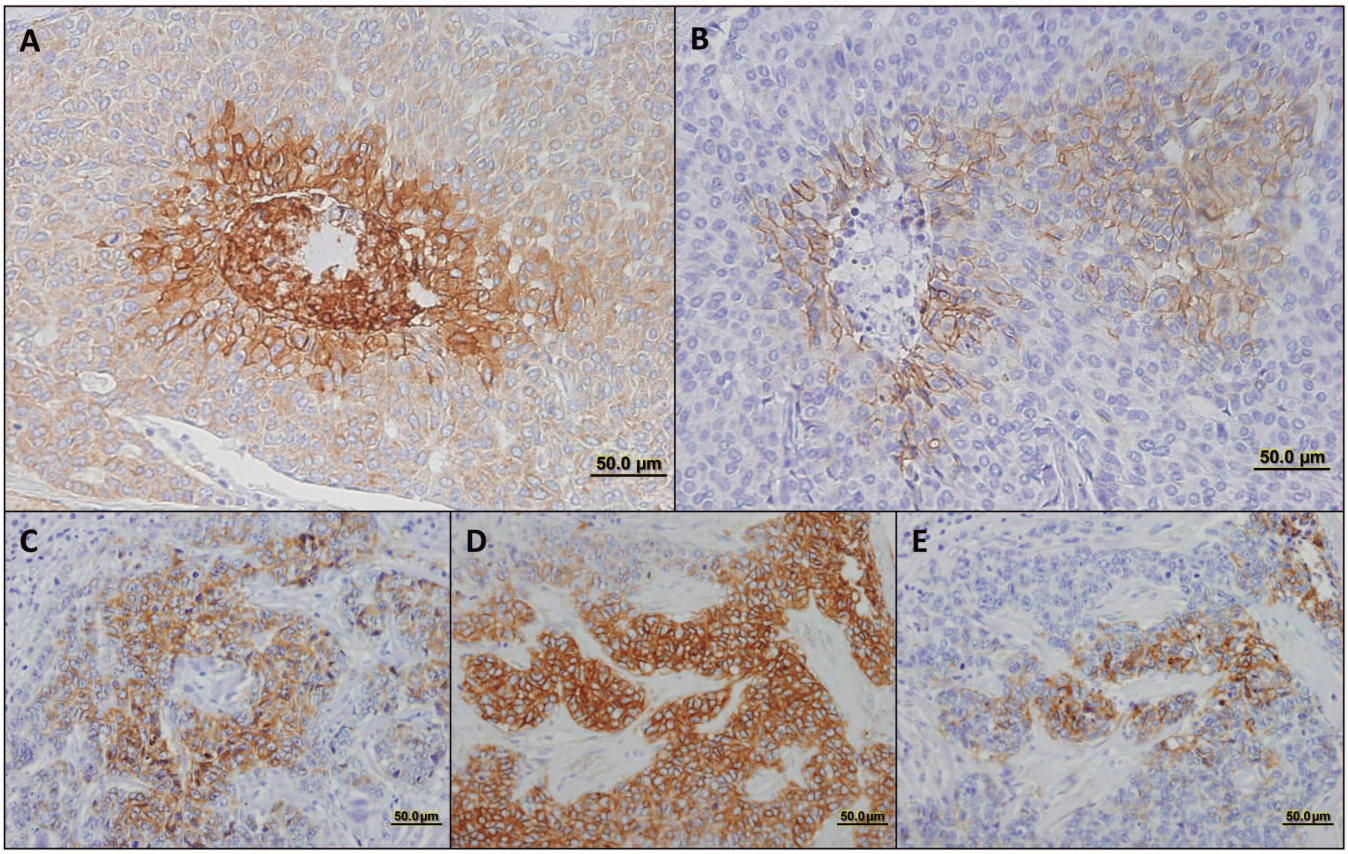

Fig. 1. Immunohistochemcial expression of glucose transporter 1 (GLUT1), carbonic anhydrase IX (CAIX) and monocarboxylate transporter 1 (MCT1), in breast carcinoma samples. GLUT1 and CAIX expression was frequently observed in perinecrotic regions (**A** and **B**, respectively). CAIX expression was usually focal and mainly restricted to tumour cells. Lower panel shows a breast ancer case simultaneously positive for MCT1 (**C),** GLUT1 (**D)** and CAIX **(E),** with staining in the same tumour region.

test, as appropriate, the threshold for significance p values being <0.05. Disease-free and overall survival curves were plotted using the method of Kaplan-Meier and data were compared using the log-rank test. The first 5 years following primary therapy was considered because recurrence rates are expected to be highest in

Table 1. Association of CAIX and GLUT1 with MCT1, MCT4 and CD147 expression in breast carcinoma samples.

	n	MCT1 Positive (%)	*p*	MCT4 Positive (%)	*p*	CD147 Positive (%)	*p*
GLUT1	106		**<0.0001**		0.3473		**0.0032**
Negative	55	1 (1.8)		4 (7.3)		2 (3.6)	
Positive	51	15 (29.4)		7 (13.7)		12 (23.5)	
CAIX	105		**<0.0001**		0.6897		**0.0005**
Negative	84	6 (7.1)		8 (9.5)		7 (8.3)	
Positive	21	10 (47.6)		3 (14.3)		8 (38.0)	

Table 2. Associations of CAIX and GLUT1 expression with clinico-pathological data from breast cancer cases.

Clinico-pathological data	GLUT1			CAIX		
	n	Positive (%)	*p*	n	Positive (%)	*p*
T size (TNM)	121		0.5218	119		**0.0034**
T1	46	23 (50.0)		45	6 (13.3)	
T2	63	25 (39.7)		63	9 (14.3)	
T3	12	6 (50.0)		11	6 (54.5)	
Histological grade	124		**0.0014**	122		**0.0263**
I	23	5 (21.7)		24	2 (8.3)	
II	55	22 (40.0)		51	6 (11.8)	
III	46	30 (65.2)		47	14 (29.8)	
Subtype	114		**0.0008**	113		**0.0050**
Luminal	78	31 (39.7)		79	8 (10.1)	
Basal-like	25	20 (80.0)		24	9 (37.5)	
HER2 overexpressing	11	3 (27.3)		10	3 (30.0)	
Estrogen receptor	124		0.1059	122		**0.0014**
Negative	45	25 (55.6)		42	14 (33.3)	
Positive	79	32 (40.5)		80	8 (10.0)	
Progesterone receptor	124		**0.0162**	122		**0.0292**
Negative	75	41 (54.7)		73	18 (24.6)	
Positive	49	16 (32.6)		49	4 (8.2)	
HER2 overexpression	123		0.5885	121		0.4556
Negative	110	51 (46.4)		109	19 (17.4)	
Positive	13	5 (38.5)		12	3 (25.0)	
EGFR	124		>0.9999	122		0.6643
Negative	115	53 (46.1)		113	20 (17.7)	
Positive	9	4 (44.4)		9	2 (22.2)	
CK5	124		0.1188	122		**0.0002**
Negative	98	42 (42.8)		97	11 (11.3)	
Positive	26	15 (57.7)		25	11 (44.0)	
CK14	121		0.0508	121		**0.0102**
Negative	114	51 (44.7)		115	18 (15.6)	
Positive	7	6 (85.7)		6	4 (66.7)	
Vimentin	106		**0.0033**	106		**0.0004**
Negative	89	38 (42.7)		88	12 (13.6)	
Positive	17	14 (82.4)		18	9 (50.0)	
Ki67	124		**0.0339**	122		0.5214
< 20%	65	24 (36.9)		63	10 (15.9)	
> 20%	59	33 (55.9)		59	12 (20.3)	
Lymph-node metastasis	117		0.6326	115		0.5840
Absent	58	24 (41.4)		55	8 (14.5)	
Present	59	27 (45.8)		60	11 (18.3)	

this period of time, especially in series with high number of ER negative cases like the one herein studied (Emens and Davidson, 2003). Cases lacking one or more of the clinico-pathological variables were not included in the specific statistical analysis.

Results

A total of 124 breast carcinoma samples, organised into TMAs (Tissue Microarrays), were assessed for GLUT1 and CAIX immunohistochemical expressions.

In general, positive GLUT1 expression was observed in both plasma membrane and cytoplasm (Fig. 1A), while CAIX expression was mainly observed in the plasma membrane, with some cases also presenting cytoplasm staining (Fig. 1B). GLUT1 expression was observed only in the epithelial cells of 1 out of 20 normal samples evaluated (5%), with a significantly higher frequency (46%) in tumour samples (57/124, p=0.0005), while CAIX expression was absent in normal tissue but was found in 18% (22/122) of breast cancer cases (p=0.0388). Importantly, the expression of both molecules was frequently observed in perinecrotic regions, as can be observed in Figure 1A,B, especially CAIX, which was usually focal and mainly restricted to tumour cells adjacent to areas of necrosis.

Importantly, when comparing the expression of the previously analysed MCT1, MCT4 and CD147 (Pinheiro et al., 2010) with GLUT1 and CAIX, we found that both MCT1 and CD147, but not MCT4, were more frequently expressed in GLUT1 and CAIX positive tumour samples (Table 1). Also, as expected, GLUT1 and CAIX were significantly co-expressed, with 81.8% (18/22) of CAIX positive cases also positive for GLUT1, versus 37.5% (36/96) GLUT1 positive in the CAIX negative group, p=0.0002. Figure 1C-E shows a breast cancer case simultaneously positive for MCT1, GLUT1 and CAIX, in the same tumour area.

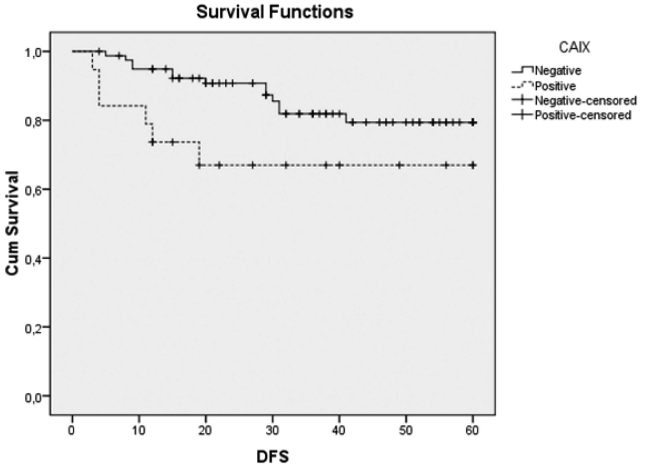

Fig. 2. Disease-free survival (DFS) curve regarding CAIX immunoreaction in breast cancer patients. Patients with positive tumours for CAIX expression show shorter disease-free survival (interrupted line) than patients with CAIX negative tumours (continuous line) (p=0.045).

Assessment of the clinico-pathological value of GLUT1 and CAIX also retrieved important results (Table 2). We found significant associations between GLUT1 expression and high grade tumours (p=0.0014), basal-like subtype (p=0.0008), absence of PR (p=0.0162), presence of vimentin (p=0.0033) and high proliferative index as measured by Ki67 expression (p=0.0339). Additionally, CAIX was associated with the majority of the clinico-pathological parameters analysed, including tumour size (p=0.0034), histological grade (p=0.0263), molecular subtype (p=0.0050), ER and PR negativity (p=0.0014 and p=0.0292, respectively), expression of CK5 (p=0.0002), CK14 (p=0.0102) and vimentin (p=0.0004).

Analysis of GLUT1 expression and patients' survival (disease-free survival and overall survival) showed no significant differences between negative and positive groups (data not shown), but, importantly, patients with CAIX positive tumours had a lower disease-free survival than patients with CAIX negative tumours (43.2 *versus* 52.4 months, respectively, p=0.045) (Fig. 2). No significant differences in overall survival were observed between the CAIX negative group and the CAIX positive group.

Discussion

Upregulation of glucose conversion into lactate, even in the presence of oxygen (Warburg effect), has been described as a possible adaptive mechanism to overcome intermittent hypoxia in pre-malignant lesions. This metabolic switch leads to an increase in acid production by cancer cells and, therefore, the need for further adaptation by means of intracellular pH regulation and resistance to extracellular acidity (Gatenby and Gilles, 2004). In this perspective, MCTs emerge as important contributors to cancer cell adaptation due to their function, on one hand, of lactate export, allowing continuous glycolysis, and, on the other hand, of tumour intracellular pH regulation and induction of extracellular acidosis, by co-transporting lactate and a proton. Although the contribution of MCTs to the glycolytic and acidic phenotype of tumours is suggested (Mathupala et al., 2007), the significance of tumour MCT expression in this context is still not clear. Thus, the main aim of the present work was to determine whether glycolytic and acid-resistant tumours, with upregulation of GLUT1 and CAIX, present a higher expression of MCTs, supporting the involvement of these transporters in the metabolic adaptations of cancer cells.

GLUT1, involved in glucose uptake, is upregulated in a variety of tumours (for a review see Macheda et al., 2005), being the hypoxia transcription factor HIF-1α the major regulator of its expression in cancer cells

(Baumann et al., 2007). HIF-1α, the master transcriptional regulator of tumour cell adaptation to hypoxic stress, activates a number of genes, many of which code for proteins involved in O_2 delivery, angiogenesis, energy preservation (including glucose transporters and glycolytic enzymes), and other processes essential to tumour cell survival, proliferation, and spread (Vaupel and Harrison, 2004). Moreover, CAIX and MCT4 are also downstream targets of HIF-1α (Wykoff et al., 2000; Ullah et al., 2006; Perez et al., 2010) and both GLUT1 and CAIX are recognised as tumour hypoxia markers (Vordermark and Brown, 2003). However, in our study, MCT4 expression was not increased in GLUT1 or CAIX positive tumours. In contrast, MCT1 was more frequently expressed in both GLUT1 and CAIX positive tumours, pointing to a hypoxia dependent upregulation of this MCT isoform, which is accompanied by co-expression of its chaperone CD147, essential for plasma membrane localization and transporter activity (Kirk et al., 2000; Wilson et al., 2005). This finding is of major importance since it supports the induction of MCT1 expression by hypoxia (Perez et al., 2010), which is contested by some groups (Ullah et al., 2006; Sonveaux et al., 2008). With these associations, one can support the role of functional MCT1 as the lactate transporter responsible for lactate efflux in highly glycolytic breast cancer cells, especially in basal-like tumours. The transport activity of MCT1 is considered one of the most important mechanisms of intracellular pH regulation (Izumi et al., 2003). Besides MCTs, carbonic anhydrases, especially CAIX, play a major role in maintenance of intracellular (and extracellular) pH levels, by contributing to the extrusion of the protons generated by the high metabolic rates of glycolytic cancer cells. At the tumour cell surface, CAIX catalyses the extracellular trapping of acid, by hydrating the cell-generated CO_2 into HCO_3^- and H^+ (Swietach et al., 2007). Thus, it is not surprising to see an association between MCT1 and both GLUT1 and CAIX, which is likely a result of the overall HIF-1α-mediated metabolic adaptations, conferring a glycolytic, acid-resistant phenotype to cancer cells.

As described previously (Chia et al., 2001; Vleugel et al., 2005), GLUT1 and CAIX were mainly observed in the vicinity of necrotic areas (a consequence of tumour hypoxia) in our tumour series, which supports the hypoxia-mediated regulation of the expression of these proteins. Both hypoxia markers were absent in normal breast tissue, but upregulated in breast tumour tissues, with expression frequencies concordant with previous reports (Kang et al., 2002; Younes et al., 1995; Hussain et al., 2007; Trastour et al., 2007; Tan et al., 2009; Chen et al., 2010). Although the clinico-pathological value of GLUT1 and CAIX in breast cancer has already been studied by others (Chia et al., 2001; Younes et al., 1995; Hussain et al., 2007; Trastour et al., 2007; Tan et al., 2009), our data strengthens the importance of these proteins as prognostic markers, especially GLUT1, which has been little explored in breast cancer (Kang et al., 2002; Younes et al., 1995; Chen et al., 2010). So far, GLUT1 has been associated with lower disease-free survival, loss of ER and PR (Kang et al., 2002) and both higher grade (Younes et al., 1995; Kang et al., 2002; Chen et al., 2010) and proliferative activity (through Ki67 expression) (Younes et al., 1995). In the present work, although we did not find associations of GLUT1 with disease-free or overall survival, GLUT1 was more frequently expressed in high grade tumours, negative for PR and with high proliferative index (Ki67). Importantly, we found GLUT1 to be more frequently expressed in basal-like tumours, as well as in vimentin positive tumours. With respect to CAIX, more data has been published in breast carcinomas, which identifies CAIX as a marker of aggressive tumour behaviour. This protein was positively correlated with larger tumour size, basal-like (Tan et al., 2009) and high grade tumours (Chia et al., 2001; Span et al., 2003; Trastour et al., 2007; Tan et al., 2009, Chen et al., 2010), loss of ER (Chia et al., 2001; Span et al., 2003; Trastour et al., 2007; Tan et al., 2009) and PR (Span et al., 2003) as well as with shorter disease-free survival (Chia et al., 2001; Hussain et al., 2007; Tan et al., 2009). Here, we support all the previous findings described by other groups, by associating CAIX expression with high histological grade, loss of ER and PR and, importantly, basal-like subtype and disease-free survival. These results suggest that the basal-like subtype tumours may be more representative of the glycolytic, acid-resistant phenotype proposed for cancer cells and this hypoxia mediated phenotype may explain, at least partly, the more aggressive phenotype of this breast carcinoma subtype.

In the present study, we investigated the expression of the key hypoxia regulated proteins GLUT1 and CAIX. Importantly, they were positively associated with the major lactate transporter, MCT1, especially in a subset of aggressive breast carcinomas (basal-like), where these proteins are more frequently expressed. Since this subtype of tumours does not have a specific molecular therapy (Matos et al., 2005), the development of therapeutic approaches targeting these particular metabolic features could be a promising strategy to be explored in the treatment of basal-like breast tumours.

Acknowledgements. We thank Dr. Vitor Carneiro (pathologist from the Hospital do Divino Espírito Santo, Ponta Delgada, São Miguel, Azores), for his help in retrieving the breast cancer samples for this study. CP and AA received fellowships from the Portuguese Foundation for Science and Technology (FCT), refs. SFRH/BD/27465/2006 and SFRH/BD/15316/2005, respectively. This work was supported by the FCT grant ref. PTDC/SAU-FCF/104347/2008, under the scope of "Programa Operacional Temático Factores de Competitividade" (COMPETE) of "Quadro Comunitário de Apoio III" and co-financed by Fundo Comunitário Europeu FEDER.

Conflict. The authors declare no conflict of interest.

Ethics. The present study has been approved by the local Ethics Committee.

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Accepted April 8, 2011

PUBLICATIONS

Paper 10

1Alpha,25-dihydroxyvitamin D_3 Induces *de novo* E-cadherin Expression in Triple-negative Breast Cancer Cells by *CDH1*-promoter Demethylation

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Abstract. *Background: The triple-negative subgroup of breast cancer includes a cluster of tumors exhibiting low E-cadherin expression (metaplastic carcinomas). In several cancer models, 1alpha,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$) induces differentiation by increasing E-cadherin expression. The Vitamin D receptor (VDR) was evaluated as a possible therapeutic target for metaplastic carcinomas and $1\alpha,25(OH)_2D_3$ effects as a differentiating agent in triple-negative breast cancer cells were assessed. Materials and Methods: Metaplastic carcinomas were assessed for VDR expression by immunohistochemistry; differences in E-cadherin expression in triple-negative breast cancer cells were evaluated by real-time PCR, western blotting and Cadherin 1 (CDH1) methylation status. Results: Most of the metaplastic carcinomas were positive for VDR expression. Furthermore, $1\alpha,25(OH)_2D_3$ promoted differentiation of MDA-MB-231 cells by inducing de novo E-cadherin expression, an effect that was time- and dose-dependent. Also, E-cadherin expression was due to promoter demethylation. Conclusion: Metaplastic carcinomas may respond to $1\alpha,25(OH)_2D_3$, since they express VDR and $1\alpha,25(OH)_2D_3$ induces de novo E-cadherin expression in breast cancer cells by promoter demethylation.*

Breast cancer is a heterogeneous disease, comprised of diverse molecular subtypes associated with different biological behaviours and clinical outcomes (1, 2). Among all breast cancer subgroups, the triple-negative basal-like type is the most aggressive, presents poor patient outcome (2) and comprises a rare cluster of carcinomas entitled metaplastic tumors (3-5). Our group and others have demonstrated that metaplastic carcinomas are distinguished by high levels of expression of classical basal-like markers, such as cytokeratin (CK) 5/6, CK14, epidermal growth factor receptor (EGFR), vimentin and P-cadherin, as well as E-cadherin down-regulation (5-7). Furthermore, patients harbouring metaplastic tumors display a worse prognosis, exhibiting lower rates of disease-free survival than those with invasive ductal carcinomas (8, 9). Due to their triple-negative phenotype, metaplastic carcinomas do not have a directed therapy. Since radiation and chemotherapy remain the only options to treat these carcinomas, intensive research on alternative therapeutic strategies is mandatory.

1Alpha,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$), the biologically active form of vitamin D, is a steroid hormone that exerts most of its biological activities by binding to a specific high-affinity receptor, the vitamin D receptor (VDR) (10). We previously reported that 56% of invasive breast carcinomas express the VDR and, among these, 56% of the cases classified as triple-negative basal-like tumors are positive for VDR expression (11), suggesting that they may be responsive to the anti-carcinogenic properties of $1\alpha,25(OH)_2D_3$. In several cancer models, $1\alpha,25(OH)_2D_3$ participates in cell growth regulation and cell differentiation (12). In breast cancer cells, it was demonstrated that $1\alpha,25(OH)_2D_3$ is able to induce cells to be more adhesive to each other, as well as to some substrates, through an increase in the expression of endogenous E-cadherin and other adhesion molecules (13). Additionally, $1\alpha,25(OH)_2D_3$ promotes the differentiation of colon cancer cells by inducing the expression of E-cadherin in VDR-expressing cells (14) and a similar result was obtained in prostate cancer with a $1\alpha,25(OH)_2D_3$ analogue (15).

These data provide good evidence for the ability of $1\alpha,25(OH)_2D_3$ to act as an epithelial differentiation-inducing agent. Therefore, the purpose of the current work was to study if the VDR could be a potential therapeutic target for

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Key Words: Vitamin D, E-cadherin, breast cancer, VDR, methylation, $1a,25(OH)_2D_3$.

metaplastic triple-negative breast carcinomas. Additionally, the *in vitro* effects of $1\alpha,25(OH)_2D_3$ as a differentiating agent in triple-negative breast cancer cell lines were evaluated.

Materials and Methods

Immunohistochemistry. A series of 12 formalin-fixed paraffin-embedded metaplastic breast carcinomas were retrieved from the archives of the Federal University of São Paulo, Brazil and from the Federal University of Santa Catarina, Brazil. The cases were collected between 1994 and 2009. Immunohistochemical staining for the VDR was performed as described elsewhere (11).

Cell culture and treatments. All the breast cancer cells (MDA-MB-231, Hs578T and BT-549, commercially available from ATCC), representative of mesenchymal triple-negative breast cancer (16, 17) were grown in complete GIBCO, Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA, USA) in the presence of 10% foetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Treatments with $1\alpha,25(OH)_2D_3$ 100 nM (Cayman Chemical, Denver, CO, USA), 5-aza-2-deoxycytidine 5 μM (5-aza-dC, Sigma, Munich, Germany), DMSO (dimethyl sulfoxide, vehicle for 5-aza-dC and Trichostatin A [TSA]) and ethanol (vehicle for $1\alpha,25(OH)_2D_3$) were performed for 72 hours, while the treatment with TSA 100 nM (Sigma) was performed only for 16 hours. Every 24 hours, the culture medium was changed and a fresh new treatment agent was added.

Western blotting. Total protein lysates were prepared from the cultured cells and the protein concentration was determined using the Bradford assay (Bio-Rad protein quantification system, Berkeley, CA, USA). Equal protein samples were separated in an 8% SDS-PAGE and the proteins were transferred onto nitrocellulose membranes (GE Healthcare Life Sciences, Chalfont St Giles, UK). For immunostaining, the membranes were blocked for non-specific binding with 5% (w/v) non-fat dry milk, in PBS containing 0.5% (v/v) Tween-20. The membranes were incubated with the primary antibodies (α-tubulin, clone DM1A, Sigma, 1:10000 for 1 hour; β-actin, clone I19, Santa Cruz [Santa Cruz, CA, USA], 1:1000 for 1 hour; E-cadherin, clone 24E10, Cell Signaling [Beverly, MA, USA], 1:1000 for 1 hour; and VDR, clone 9A7γE10.4, Calbiochem [Darmstadt, Germany], 1:400 overnight), followed by four 5 min washes in PBS/Tween-20; then they were incubated with horseradish peroxidase-conjugated secondary antibodies (all 1:1000, Santa Cruz) for 60 min. The membranes were then washed six times more for 5 min and the proteins detected using the ECL detection system (GE Healthcare Life Sciences).

RNA extraction, cDNA synthesis and quantitative real-time PCR. The RNA was extracted from the breast cancer cells using TRIzol® reagent (Invitrogen) and cDNA was synthesised from 1 μg of RNA, using an Omniscript Reverse Transcription kit (Qiagen, Düsseldorf, Germany), following the manufacturer's instructions. Real-time PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA, USA), using 1 μL of cDNA and in accordance with the manufacturer's protocol. The TaqMan Gene Expression Assays used were Hs01023895_m1 (for *CDH1* [Cadherin 1], Applied Biosystems) and TaqMan PreDeveloped Assay Reagents Human GAPDH (for *GAPDH* [Glyceraldehyde 3-phosphate dehydrogenase], Applied Biosystems). The reactions were performed using standard cycle parameters and relative transcript levels were determined using human *GAPDH* as an internal reference. Differences between samples were determined using the Quantitation–Relative Standard Curve method.

DNA extraction and CDH1 promoter methylation analysis. The DNA was extracted from the breast cancer cell lines using an ULTRAPrep Genomic DNA Blood and Cell Culture Kit (AHN Biotechnologie, Nordhausen, Germany), according to the manufacturer's instructions. Bisulfite treatment was performed on 300 ng of DNA, using an EpiTect Bisulfite kit (Qiagen) following the manufacturer's guidelines. Unmethylated cytosines were converted to uracil, whereas methylated ones remained unmodified. The 12 CpG sites (cytosine-phosphate-guanine) within the 90 base pairs upstream of the *CDH1* translation start site (ATG) were analysed, as described elsewhere (18).

Immunofluorescence. The cells were seeded on coverslips and fixed with formaldehyde 4% (v/v) for 30 min The coverslips were washed three times with PBS for 5 min, followed by incubation with 50 mM NH4Cl in PBS for 10 min. Following another set of three 5 minute washes with PBS, the coverslips were incubated with Triton X-100 0.2% (v/v) for 5 min and washed with PBS three times for 5 min. Subsequently, they were blocked for non-specific binding with BSA 5% in PBS, containing 0.5% (v/v) Tween-20, for 30 min and incubated with the primary antibody for E-cadherin (Zymed, San Francisco, CA, USA, clone HECD1, 1:100) for 1 hour. After three 5 minute washes with PBS, the coverslips were incubated with a goat anti-mouse secondary antibody (Alexa Fluor 594, 1:500, Invitrogen), washed with PBS for 3 times 5 min and mounted using Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA).

Transfection with siRNA for VDR. MDA-MB-231 cells (2.5×10^5 cells) were cultured in 6-well plates for 24 hours. For each well, 150 nmol of siRNA against VDR (Hs_VDR_8 FlexiTube siRNA, Qiagen) or control siRNA (Qiagen) was used according to the manufacturer's instructions. After 5 hours of incubation, the cell medium was replaced and the cells were treated with $1\alpha,25(OH)_2D_3$ 100 nM and ethanol. The evaluation of siRNA efficiency occurred 48 hours after transfection.

Statistical analysis. Differences between groups were assessed using Student's *t*-test. Differences with *p*-values <0.05 were considered statistically significant. All the presented results are representative of at least three independent experiments, unless stated otherwise.

Results

VDR expression in metaplastic breast carcinomas. Out of the 12 metaplastic breast carcinomas, 8 cases (66.7%) were positive for the expression of VDR (Figure 1).

VDR expression in triple-negative breast cancer cell lines. By western blotting, it was shown that all the cell lines studied were positive for VDR expression. The MDA-MB-231 and BT-549 cells seem to be more sensitive to $1\alpha,25(OH)_2D_3$, as in these cells there was a clear increase in VDR expression upon hormonal treatment (Figure 2).

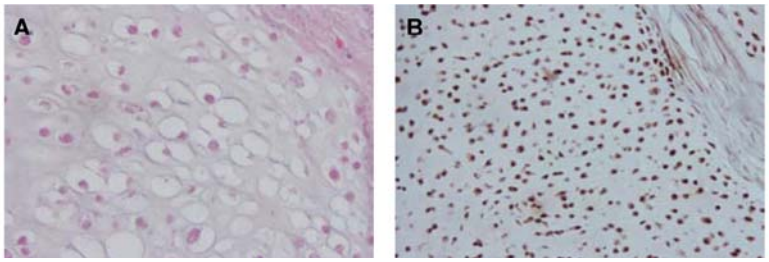

Figure 1. *H&E, magnification ×630 (A) and VDR, magnification ×400 (B) staining in a case of metaplastic breast carcinoma.*

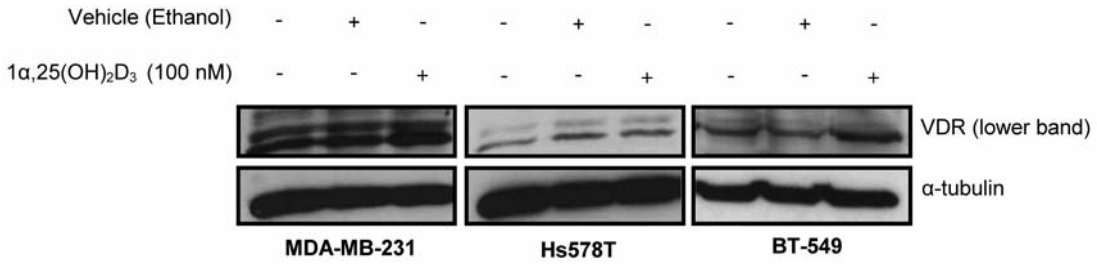

Figure 2. *Western blot of VDR expression in MDA-MB-231, Hs578T and BT-549 breast cancer cell lines.*

Effect of 1α,25(OH)$_2$D$_3$ on the expression of E-cadherin. A *de novo* expression of E-cadherin, by western blotting, was observed upon 1α,25(OH)$_2$D$_3$ treatment in the MDA-MB-231 cells (Figure 3A). As presented in Figure 3B, the expression of E-cadherin was dependent on the duration of treatment. The protein expression was first detected at 24 hours and increased with time. With 72 hours of treatment the E-cadherin expression level was dependent on the dose of 1α,25(OH)$_2$D$_3$ and was identified even with the very low dose of 1 nM (Figure 3C).

In the MDA-MB-231 cells, 1α,25(OH)$_2$D$_3$ was a potent inducer of *CDH1* mRNA expression, displaying more than 10-fold induction, compared with the control ($p<0.01$) (Figure 4A). Furthermore, the level of expression induced by 1α,25(OH)$_2$D$_3$ was 2-fold higher than that produced by the demethylating agent 5-aza-dC alone and 3-fold higher than that induced by the histone deacetylation (HDAC) inhibitor agent TSA alone. However, both agents displayed an additive effect to 1α,25(OH)$_2$D$_3$, the highest levels of expression being induced when the three drugs were combined. These results were also confirmed by the protein expression (Figure 4A). In the BT-549 cells, 1α,25(OH)$_2$D$_3$ was unable to induce E-cadherin expression on its own. However, in the Hs578T cells CDH1 expression was significantly induced upon 1α,25(OH)$_2$D$_3$ treatment (Figure 4B). Furthermore, in both cell lines *CDH1* mRNA expression was induced upon treatment with 5-aza-dC. Interestingly, 1α,25(OH)$_2$D$_3$ seemed to display an additive effect when administered with both 5-aza-dC and TSA.

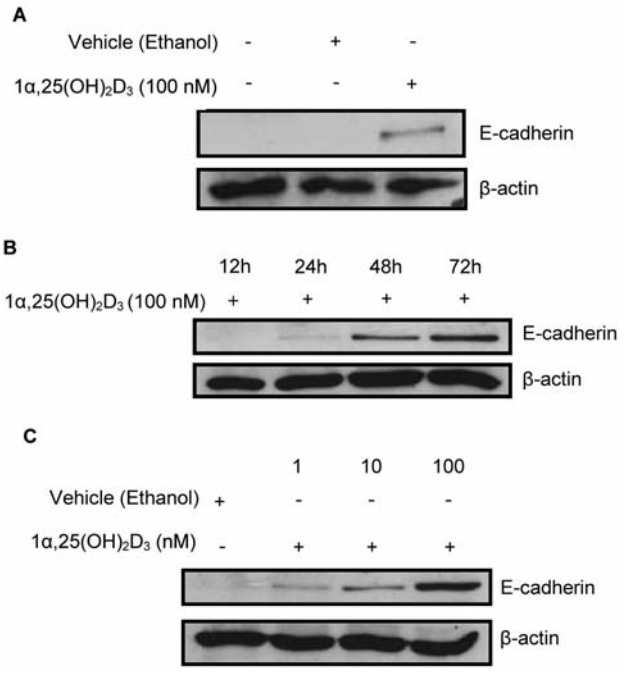

Figure 3. *Effect of 1α,25(OH)$_2$D$_3$ on E-cadherin expression in MDA-MB-231 breast cancer cells treated for 72 hours (A) or for various times (B) or at various dose rates (C), and assessed by western blotting.*

Again, the highest levels of CDH1 expression were achieved whenever all the agents were added together and, in this case, the BT-549 cells were more responsive than the

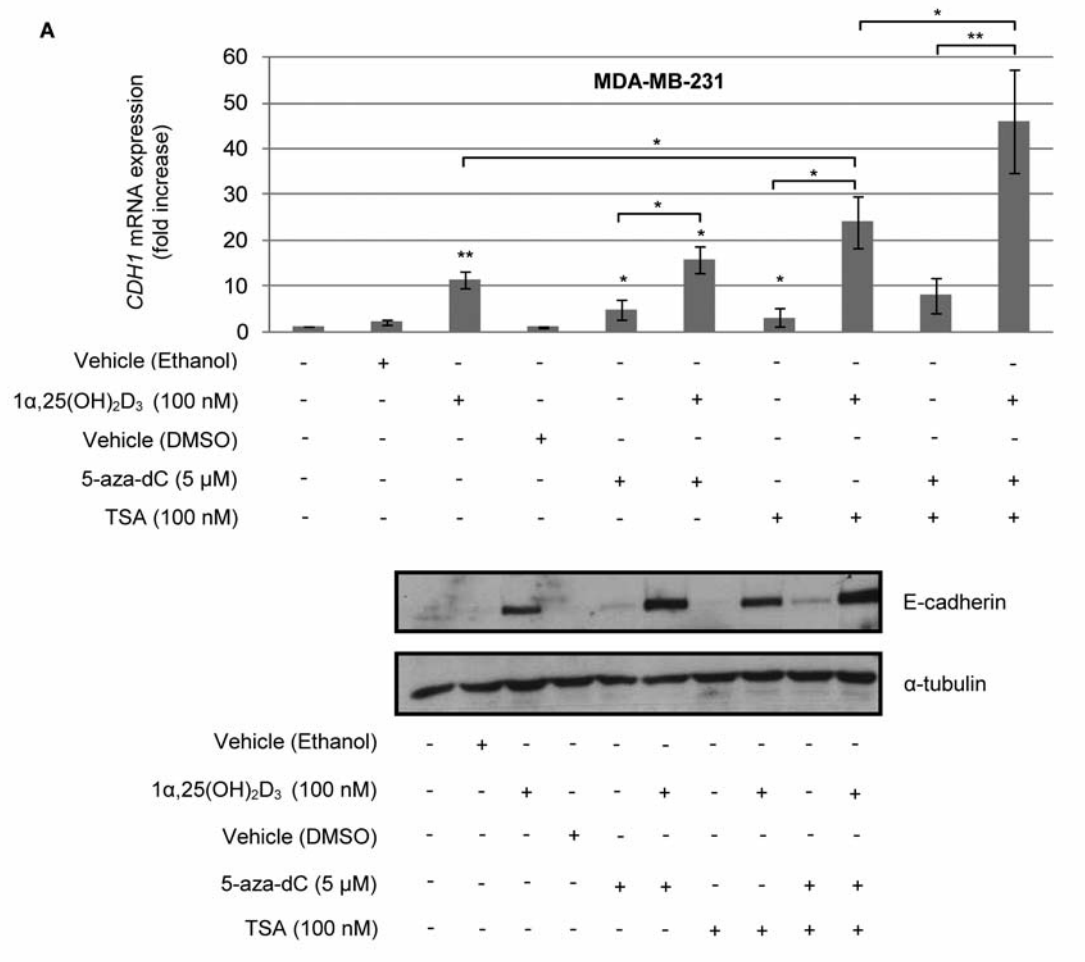

Figure 4. *Continued*

Hs578T cells, which corroborated the VDR expression results. Furthermore, these results were confirmed by the protein expression (Figure 4B and 4C).

As shown by immunofluorescence in Figure 5, upon treatment with 1α,25(OH)$_2$D$_3$, the MDA-MB-231 cells exhibited expression of E-cadherin at the plasma membrane. In contrast, the expression of E-cadherin induced by 5-aza-dC alone was granular and dispersed throughout the cytoplasm. However, when these cells were treated with both agents, the E-cadherin expression was located at the membrane.

Mediation of 1α,25(OH)$_2$D$_3$-induced expression of E-cadherin. Since 1α,25(OH)$_2$D$_3$ alone induced E-cadherin expression at the protein level only in the MDA-MB-231 cells, the experiments using *VDR* knockdown with siRNA were only conducted in this cell line. Upon silencing of the *VDR* in the MDA-MB-231 cells, the E-cadherin expression after hormonal treatment was abrogated (Figure 6).

Mechanism of E-cadherin expression. Upon 1α,25(OH)$_2$D$_3$ treatment, partial demethylation of the *CDH1* promoter in the MDA-MB-231 cells was observed (Figure 7). Demethylation was detected in 7 out of the 12 CpG sites analysed.

Discussion

The majority of the metaplastic breast carcinomas studied were positive for VDR expression, suggesting that they might be responsive to treatment with 1α,25(OH)$_2$D$_3$. In addition, 67% of the tumors had previously been characterised as negative for E-cadherin expression and 83.3% exhibited vimentin expression (unpublished results), showing that these tumors were indeed undifferentiated and could benefit from the differentiation-inducing properties of 1α,25(OH)$_2$D$_3$ treatment. In the *in vitro* model, 1α,25(OH)$_2$D$_3$ induced a *de novo* E-cadherin (epithelial differentiation marker) expression in the triple-negative MDA-MB-231 breast cancer cell line. This is an important finding, given the major role of E-

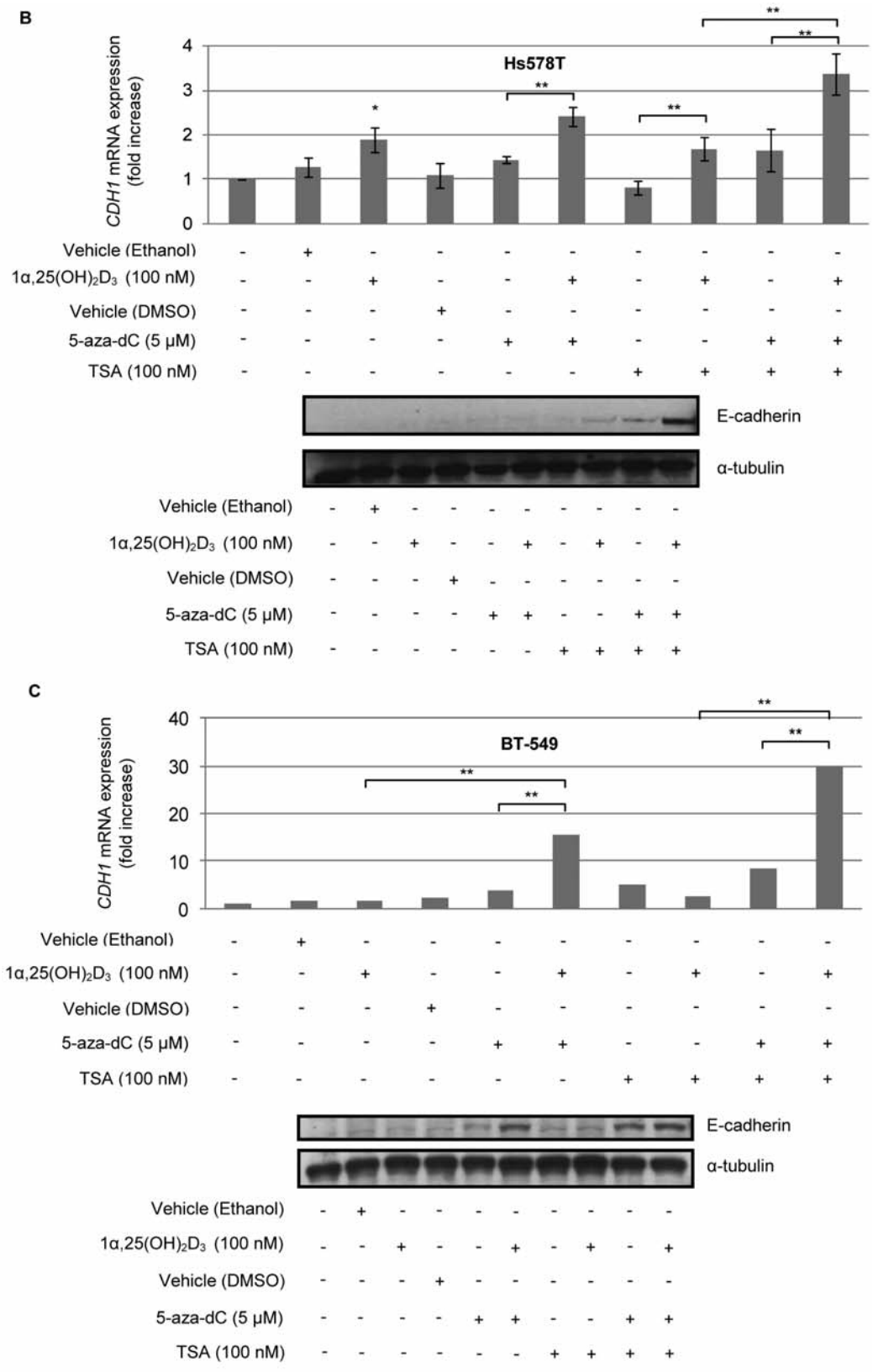

Figure 4. *Effect of 1α,25(OH)$_2$D$_3$, 5-aza-dC and TSA on CDH1 mRNA expression and E-cadherin expression in MDA-MB-231 (A), Hs578T (B) and BT-549 (C) breast cancer cells (*p<0.05, **p<0.01).*

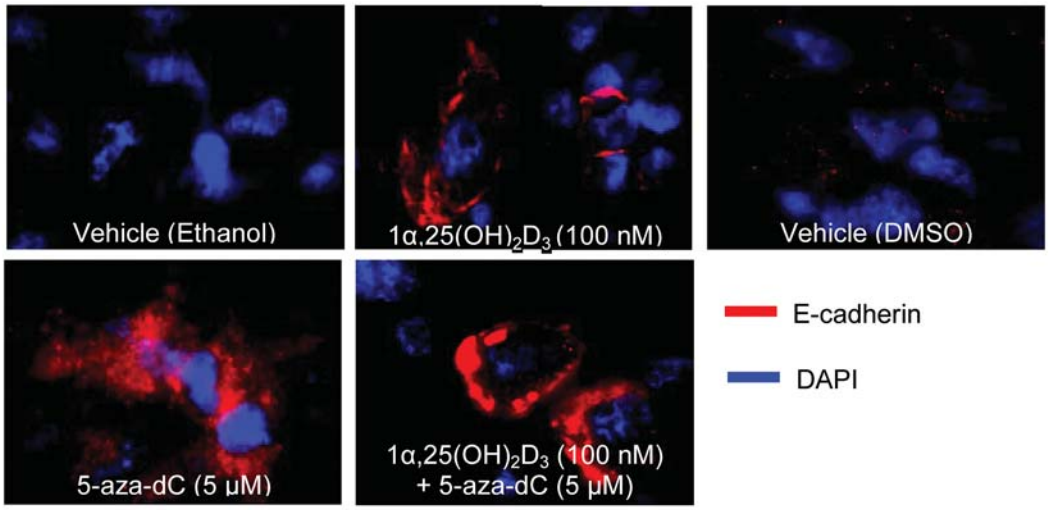

Figure 5. *Immunofluorescence of E-cadherin expression in MDA-MB-231 breast cancer cells (magnification ×400).*

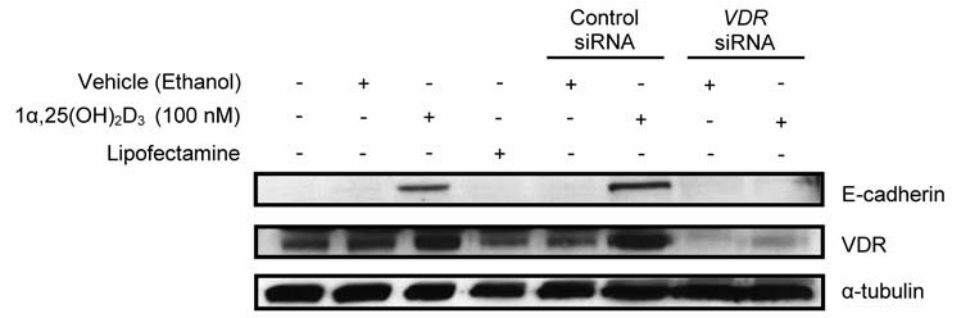

Figure 6. *Effect on E-cadherin expression induced by 1α,25(OH)$_2$D$_3$ of VDR knockdown by siRNA in MDA-MB-231 cells.*

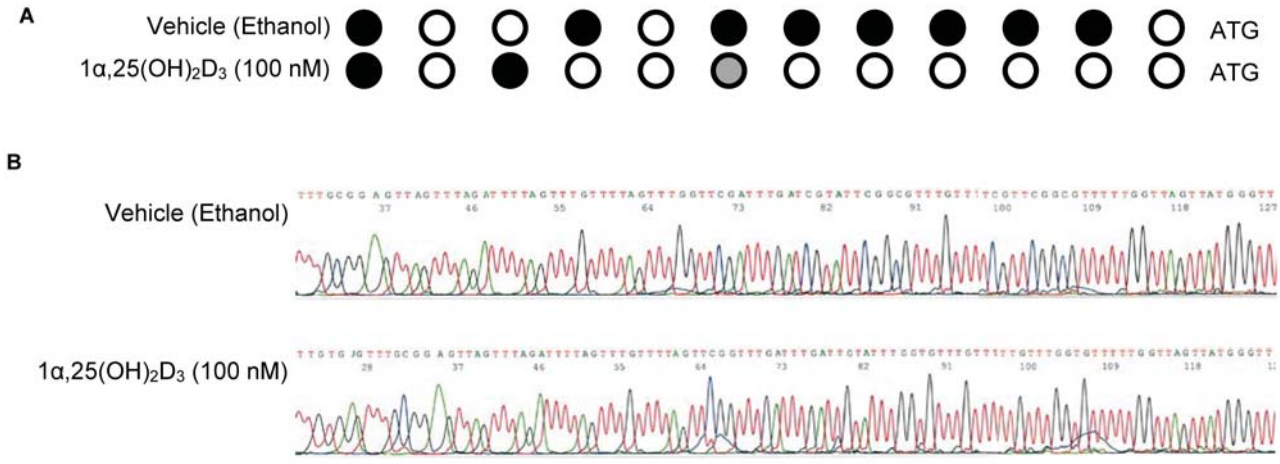

Figure 7. *Methylation analysis of CDH1 promoter in MDA-MB-231 breast cancer cells.* ● *– methylation,* ◍ *– hemimethylation,* ○ *– demethylation (A); Example of DNA sequences treated with ethanol or 1α,25(OH)$_2$D$_3$ (B).*

cadherin as a tumor suppressor protein in lobular breast carcinomas and other cancer models (19, 20) and since down-regulation of E-cadherin is required to initiate breast cancer metastatic growth (21). Furthermore, this effect was dependent on the duration of treatment and the quantity of $1\alpha,25(OH)_2D_3$ supplied to the cells. As far as we know, this is the first study demonstrating the *de novo* induction of E-cadherin expression in breast cancer cells by $1\alpha,25(OH)_2D_3$ due to *CDH1* promoter demethylation, although it has been reported that $1\alpha,25(OH)_2D_3$ can augment the expression of endogenous E-cadherin in mammary tumor cells (13). In addition, it has been demonstrated that a $1\alpha,25(OH)_2D_3$ analogue, increased the expression of E-cadherin in prostate cancer cells (15). In colon carcinoma cells, $1\alpha,25(OH)_2D_3$ is also known to promote differentiation by inducing E-cadherin expression and other adhesion proteins, an effect only observed in VDR positive cells (14). Likewise, in the MDA-MB-231 cells, E-cadherin expression was dependent on the presence of the VDR, suggesting it could mediate this effect.

In MDA-MB-231 cells, *CDH1* trancription is silenced due to promoter methylation (22). Interestingly, the levels of CDH1 expression upon $1\alpha,25(OH)_2D_3$ treatment in the MDA-MB-231 cells were 2- and 3-fold higher than those induced by the demethylating agent 5-aza-dC and by the HDAC inhibitor TSA, respectively, while the combination of $1\alpha,25(OH)_2D_3$ with either of these molecules promoted an additive effect, which was further confirmed by the protein expression. In gastric cancer cells, $1\alpha,25(OH)_2D_3$ has been shown to work in synergy with 5-aza-dC and TSA (23), thus supporting the effect obtained in the present study. Additionally, in colon cancer cells with silenced HDAC3, E-cadherin expression increased upon treatment with $1\alpha,25(OH)_2D_3$ (24), a result that mimics that observed in the MDA-MB-231 cells upon treatment with TSA and $1\alpha,25(OH)_2D_3$. In the other cells tested (Hs578T and BT-549) the results were not so encouraging when $1\alpha,25(OH)_2D_3$ was used alone; however, CDH1/E-cadherin expression was detectable when the cells were treated with $1\alpha,25(OH)_2D_3$ together with 5-aza-dC or TSA.

Also remarkably, the $1\alpha,25(OH)_2D_3$ treatment promoted the correct localisation of E-cadherin at the cell membrane in the MDA-MB-231 cells, suggesting a functional adhesion molecule, unlike the granular and dispersed pattern of expression induced by treatment with 5-aza-dC, which is suggestive of a non-functional protein. Similarly, in colon carcinoma, upon $1\alpha,25(OH)_2D_3$ treatment, E-cadherin expression was observed at the cell membrane (14). However, this $1\alpha,25(OH)_2D_3$ effect on E-cadherin induction is not exclusive of disease settings, as in normal keratinocytes, the treatment with $1\alpha,25(OH)_2D_3$ stimulates the assembly of adherens junctions, assessed by translocation of E-cadherin to the cell membrane (25). Surprisingly, when the MDA-MB-231 cells were treated with both $1\alpha,25(OH)_2D_3$ and 5-aza-dC, the effect induced by $1\alpha,25(OH)_2D_3$ prevailed over the 5-aza-dC-induced effect and there was a rescue of E-cadherin expression back to the membrane, hinting that $1,25(OH)_2D_3$ is indeed inducing not only the expression of E-cadherin, but, apparently, it is also important for the correct membrane localisation of the protein as a cell-cell adhesion molecule. Unlike the current results, 5-aza-dC was found to be necessary to sensitise leukaemia cells to differentiate in response to $1\alpha,25(OH)_2D_3$ treatment (26).

For the first time, $1\alpha,25(OH)_2D_3$ was found to promote partial *CDH1* promoter demethylation, suggesting that $1\alpha,25(OH)_2D_3$ can work as a demethylating agent in MDA-MB-231 breast cancer cells. To the best of our knowledge, only one study has correlated $1\alpha,25(OH)_2D_3$ with methylation and reported that it induced methylation of *CYP27B1* (the enzyme responsible for its synthesis) and, thus, silenced its expression (27). In colon cancer cells, where $1\alpha,25(OH)_2D_3$ induces E-cadherin expression, a new mechanism involving phosphoinositide signalling was recently proposed (28). Also in colonic cancer cells, a novel mechanism involving $1\alpha,25(OH)_2D_3$ in epigenetic events was reported, where the knockdown of *KDM6B*/*JMJD3*, a histone demethylase induced by $1\alpha,25(OH)_2D_3$, down-regulated E-cadherin expression (29). Studies addressing the importance of these mediators in breast cancer are still lacking.

In summary, the majority of metaplastic carcinomas examined were positive for VDR expression, hinting that this rare type of aggressive cancer may be responsive to the antitumor effects of $1\alpha,25(OH)_2D_3$. Furthermore, $1\alpha,25(OH)_2D_3$ induced the *de novo* expression of the epithelial differentiation marker E-cadherin in the highly metastatic, triple-negative MDA-MB-231 breast cancer cell line. To the best of our knowledge, this is the first report of the *de novo* induction of E-cadherin in breast cancer cells by $1\alpha,25(OH)_2D_3$ due to *CDH1* promoter demethylation, therefore, revealing a novel mechanism for the action of $1\alpha,25(OH)_2D_3$ in breast cancer cells. The induction of differentiation promoted by $1\alpha,25(OH)_2D_3$ in triple-negative metaplastic breast cancer may decrease the aggressiveness of this subtype of mammary carcinomas and improve patient outcome, but further studies are necessary to confirm this hypothesis.

Acknowledgements

This study was supported by five research grants (Nair Lopes: SFRH/BD/39208/2007; Joana Carvalho: SFRH/BD/44074/2008; Cecília Durães: SFRH/BPD/62974/2009; Bárbara Sousa: SFRH/BD/69353/2010; Madalena Gomes: PIC/IC/83264/2007; José Luis Costa: SFRH/BPD/20370/2004) and by a scientific project (PIC/IC/83264/2007), all financed by Fundação para a Ciência e Tecnologia (Portugal). Salary support was provided for Joana Paredes and Carla Oliveira by the POPH – QREN/Type 4.2,

European Social Fund and Portuguese Ministry of Science and Technology. IPATIMUP is an Associate Laboratory of the Portuguese Ministry of Science, Technology and Higher Education and is partially supported by Fundação para a Ciência e Tecnologia.

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Received September 28, 2011
Revised November 10, 2011
Accepted November 11, 2011

PUBLICATIONS

Paper 11

Human Molecular Genetics, 2013, Vol. 22, No. 1 **84–95**
doi:10.1093/hmg/dds405
Advance Access published on October 3, 2012

Loss of WNK2 expression by promoter gene methylation occurs in adult gliomas and triggers Rac1-mediated tumour cell invasiveness

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Received July 11, 2012; Revised and Accepted September 21, 2012

The gene encoding protein kinase *WNK2* was recently identified to be silenced by promoter hypermethylation in gliomas and meningiomas, suggesting a tumour-suppressor role in these brain tumours. Following experimental depletion in cell lines, WNK2 was further found to control GTP-loading of Rac1, a signalling guanosine triphosphatase involved in cell migration and motility. Here we show that *WNK2* promoter methylation also occurs in 17.5% (29 out of 166) of adult gliomas, whereas it is infrequent in its paediatric forms (1.6%; 1 out of 66). Re-expression of WNK2 in glioblastoma cells presenting *WNK2* gene silencing reduced cell proliferation *in vitro*, tumour growth *in vivo* and also cell migration and invasion, an effect correlated with reduced activation of Rac1. In contrast, when endogenous WNK2 was depleted from glioblastoma cells with unmethylated *WNK2* promoter, changes in cell morphology, an increase in invasion and activation of Rac1 were observed. Together, these results validate the *WNK2* gene as a recurrent target for epigenetic silencing in glia-derived brain tumours and provide first mechanistic evidence for a tumour-suppressing role of WNK2 that is related to Rac1 signalling and tumour cell invasion and proliferation.

INTRODUCTION

Gliomas are the most common primary adult and paediatric brain tumours, and are composed by distinct histological subtypes and World Health Organization (WHO) malignancy grades (1,2). Astrocytic tumours are the most frequent histological subtype and can be divided into pilocytic astrocytomas (WHO grade I), diffuse astrocytomas (WHO grade II), anaplastic astrocytomas (WHO grade III) and glioblastomas (WHO grade IV) (1). Oligodendrogliomas are the second most frequent subtype, and can be subdivided into low-grade oligodendrogliomas (WHO grade II) and anaplastic

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oligodendrogliomas (WHO grade III). In adults, glioblastoma (WHO grade IV) is not only the most aggressive type, with a mean survival of ~16 months, but also the most frequent, accounting for ~50% of all gliomas and ~15% of all primary brain tumours (1). In contrast to adults, in children, the most common form is pilocytic astrocytomas. Despite the rarity of malignant gliomas in the paediatric context, they are one of the leading causes of cancer-related deaths in children. A growing body of evidence demonstrates that paediatric malignant gliomas not only exhibit distinct clinicopathological features but also harbour a different genetic profile (3). One of the major causes of the high mortality of glioblastoma is their infiltrative and invasive property that hampers complete surgical excision, with consequent tumour regrowth and recurrence (2,4).

Cell invasion is known to be regulated by Rho family guanosine triphosphatases (GTPases), which control the dynamics of the actin cytoskeleton (5). Meanwhile, two different types of tumour cell motility have been identified in 3D matrices (6,7). A RhoA-dependent bleb-associated motility (amoeboid migration mode) exists that does not require pericellular proteolysis and generates hydrostatic pressure, which squeezes tumour cells through pre-existing gaps in the matrix. In contrast, an elongated mechanism of cell motility (mesenchymal migration mode) is associated with Rac1-dependent polymerization of actin filaments and induces branching of existing actin filaments that results in cell-surface structures, known as lamellipodia or membrane ruffles.

WNK2 is a member of the WNK (with-no-lysine [K]) subfamily of protein kinases (8,9), which is predominantly expressed in the brain, heart muscle, small intestine and colon (10). Functional analysis has revealed that reducing WNK2 expression by RNA interference in epithelial cell lines resulted in increased GTP-loading of Rac1 and concomitant stimulation of the Rac1-effector kinase PAK1 (p21-activated kinase). This effect activates a signalling cross-talk, in which PAK1 phosphorylates MAPK/extracellular signal-regulated kinase (ERK) 1 (MEK1) at serine 298, thereby increasing the efficiency with which MEK1 activates ERK1/2 upon growth factor stimulation (11).

Recently, *WNK2* has been proposed to act as a specific tumour-suppressor gene for brain tumours. First, an unbiased genome-wide approach to mapping non-random and tumour type-specific epigenetic gene silencing identified the *WNK2* gene in 29 of 31 infiltrative gliomas (12), the underlying mechanism being promoter methylation. Second, *WNK2* was reported to be aberrantly methylated in 83 and 71% of grade II and III meningiomas, respectively, but rarely in a total of 209 tumours from 13 other tumour types (13).

In this study, we detected *WNK2* silencing in ~17.5% of adult gliomas and describe that experimental manipulation of WNK2 expression levels affected tumorigenic growth properties of glioblastoma cell lines and conferred increased invasiveness in a Rac1-dependent manner.

RESULTS

Methylation of the human *WNK2* gene promoter in glioblastoma

Based on previous reports showing that the *WNK2* gene is a recurrent target for epigenetic silencing in oligodendrogliomas and meningiomas (12,13), we developed a methylation-specific polymerase chain reaction (PCR) (MSP) to test for the prevalence of *WNK2* promoter methylation in adult and paediatric malignant glioma cases. Among 61 childhood high-grade gliomas, only one case (1.6%) was identified with *WNK2* promoter methylation, corresponding to an 18-year-old patient. In contrast, we found that 29 out of 166 adult glioma samples (17.5%) showed *WNK2* methylation (Fig. 1A). The majority of samples were glioblastoma ($n = 115$; 18.3% methylated). In order to further confirm the MSP results in six adult glioblastoma cases (three methylated and three unmethylated), we performed bisulphite sequencing

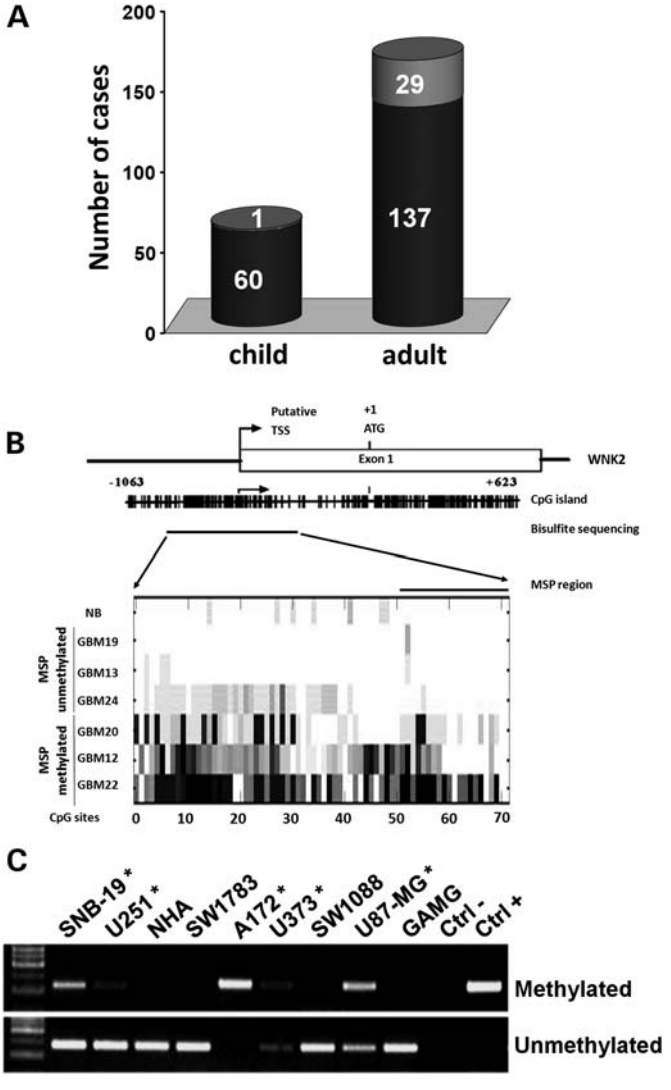


Figure 1. Methylation of the human *WNK2* gene promoter in gliomas. (**A**) Graphic representation of the analysis of 166 adult and 61 paediatric gliomas by MSP at the WNK2 promoter. (**B**) Bisulphite sequencing of the WNK2 CpG island in three MSP-positive, three MSP-negative adult glioblastoma samples and normal brain (NB). Shown are a graphical representation of the CpG island with 74 CG dinucleotides (top) and a grey-scale-coded frequency chart of methylated positions in the seven samples. (**C**) MSP analysis of the *WNK2* gene promoter in the eight indicated glioblastoma cell lines and NHA. The cell lines found to be promoter-methylated are marked by an asterisk.

of their *WNK2* promoter region and were able to fully validate the observed methylation status at nucleotide-level resolution (Fig. 1B and Supplementary Material, Fig. S1).

No significant association was observed between *WNK2* promoter methylation and clinicopathological data such as age, gender, cellular lineage, histological subtype, malignancy grade or patient survival (Supplementary Material, Table S1). Additionally, we made use of a glioblastoma data set from The Cancer Genome Atlas (TCGA) containing data for both methylation and mRNA expression of *WNK2*. A significant correlation between the downregulation of *WNK2* expression and the presence of promoter methylation was found; however, similar to our data, there was no correlation between *WNK2* methylation and clinicopathological data (Supplementary Material, Table S2). Interestingly, when only mRNA expression was considered, a significant correlation was found between *WNK2* downregulation and higher age or lower survival time (Supplementary Material, Table S2 and Fig. S2).

These results suggested that other mechanisms may exist besides the described promoter methylation, which lead to the downregulation of *WNK2* expression. In fact, a genome-wide sequencing of 22 glioblastoma identified one case with the missense mutation A1267T (14). In order to test the hypothesis that this mutation could be an alternative genetic event, we determined the frequency of this mutation in 48 primary glioblastomas (12 methylated and 36 unmethylated). None of the 48 glioblastomas exhibited the A1267T *WNK2* mutation (data not shown).

In order to select appropriate models to assess the cellular effects of WNK2 silencing, eight glioblastoma cell lines (SNB-19, U87-MG, U251, U373, SW1783, SW1088, A172, GAMG) and a cell line derived from normal human astrocytes (NHA) were also characterized by MSP for their *WNK2* promoter methylation status. Promoter methylation was observed in cell lines SNB19, U87-MG, U251, U373 and A172 (Fig. 1C), the latter presenting the strongest methylation signal, confirming previously described data (12).

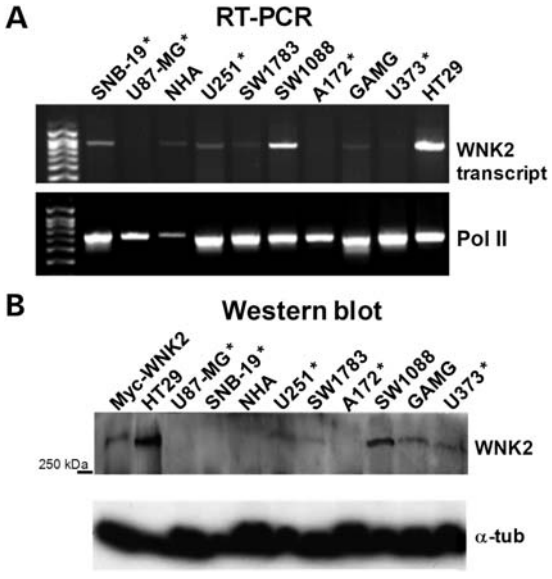


Figure 2. Expression level of endogenous WNK2 in the indicated cell lines. (**A**) Detection of WNK2 transcript by RT-PCR, including as positive control the previously reported expression in colorectal HT29 cell line (11). (**B**) Detection of WNK2 protein by western blot, including as positive controls lysates from HEK293 transfected with Myc-tagged WNK2 and HT29 cells. The cell lines shown to be promoter-methylated are marked by an asterisk.

WNK2 expression in different glioblastoma cell lines

Next, we characterized the above-mentioned cell lines for their expression levels of WNK2 transcript (Fig. 2A) and protein (Fig. 2B). In general, we found that WNK2 expression was absent or low in cell lines with promoter methylation, including A172 cells in which we showed previously that WNK2 expression can be reactivated after treatment with demethylation drugs (12). In contrast, WNK2 expression was clearly detectable in all non-methylated cell lines, among which SW1088 cells expressed the highest levels of WNK2 transcript and protein. We thus selected two cell lines as models for the subsequent experimental manipulation of WNK2 expression levels: SW1088 cells that express endogenous WNK2 from an unmethylated promoter and A172 cells that express no WNK2 due to promoter methylation.

Depletion of endogenous WNK2 increases growth and invasion of SW1088 cells

Expression of endogenous WNK2 in SW1088 cells was inhibited by RNA interference. First, stable cell lines expressing either a control short hairpin RNA (shRNA) or one of two distinct WNK2-specific shRNAs (shW2.2 and shW2.3) were selected (Fig. 3A). Clones shW2.2 and shW2.3 were selected, which revealed distinct degrees of WNK2 depletion. In addition, transient transfection of parental SW1088 cells with small interfering RNAs (siRNAs) allowed downregulation of WNK2 expression in ~50% of the cells (Fig. 3B).

The soft agar colony formation assay indicates anchorage-independent growth and demonstrated a significant ($P < 0.05$) increase in the number of the colonies formed by the stable shW2-transfected cells (Fig. 4A).

During the selection of the shW2 clones, we also observed differences in morphology and actin cytoskeleton organization (Fig. 4B). Unlike parental SW1088 and shCtrl cells, shWNK2.2 cells and especially shWNK2.3 cells showed a more flat spread-out appearance and displayed pronounced cytoplasmic protrusions consistent with lamellipodia formation. Immunofluorescence staining for actin and endogenous Rac1 showed increased accumulation at the plasma membrane (Fig. 4C).

These observations prompted us to analyse cell migration properties in wound-healing assays. First, parental SW1088 cells were transiently transfected with control or siWNK2 oligonucleotides. Approximately 48 h after siRNA transfection, scratch wounds were made across the cell monolayer and monitored by time-lapse photography for 24 h (Fig. 5A). Under these conditions, the depletion of WNK2 reached ~50% (Fig. 3B), and an increase in wound closure was observed.

Cell invasion and migration are known to be regulated by Rho family GTPases, which control the dynamics of the actin cytoskeleton (5), and our previous functional analysis of WNK2 in epithelial cells has demonstrated that depletion of WNK2 expression resulted in increased GTP-loading

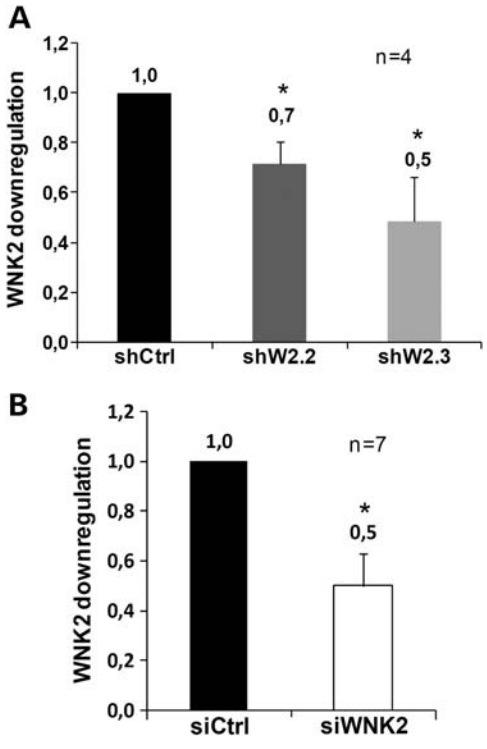


Figure 3. Efficiency of WNK2 depletion in SW1088 cells by RNA interference. (**A**) Cells were transfected with control or one of two different WNK2-specific shRNA vectors, and individual cell clones selected in the presence of puromycin. The resulting WNK2 expression levels were determined and graphically displayed ($n = 4$). (**B**) SW1088 cells were transiently transfected ($n = 7$) with control (siCtrl) or WNK2-specific (siWNK2) siRNAs oligonucleotides and remaining WNK2 transcript levels analysed by RT-PCR after 48 h. Statistically significant differences are indicated as $^{*}P < 0.05$.

of Rac1 and concomitant reduction in active RhoA (11). We thus confirmed the occurrence of an increase in Rac1 activity and a concomitant reduction in RhoA active levels in siWNK2-transfected SW1088 cells (Fig. 5B and C). These data indicated increased cell migration capacity upon the downregulation of WNK2 expression.

In order to study the motile and invasive cell properties in a more homogenous cell population, the stable shW2 clones were first investigated by *in vivo* time-lapse microscopy. Migration experiments confirmed that the shW2.2 and shW2.3 clones exhibited increased ability in closing scratch wounds (Fig. 6A and Supplementary Material, Movies M1 and M2). The *in vivo* live-cell observations further revealed that shWNK2.2 and shWNK2.3 cells presented higher cell motility and formed larger lamellipodia protrusions (Fig. 6B and Supplementary Material, Movies M3 and M4).

Based on these findings, the clones were analysed in *Matrigel* invasion assays. An increase in the number of cells that passed the membrane was observed comparing control with shW2.2 and shW2.3 cells (Fig. 6C), and this increase was proportional to the levels of active Rac1 measured in these cells (Fig. 6D). Thus, clones expressing lower WNK2 levels showed higher invasiveness compared with parental and shCtrl SW1088 cells.

Together, these data demonstrate that the downregulation of WNK2 expression in glioblastoma cells increased their cell migration and invasion capacity with a concomitant increase in Rac1 activity.

Re-expression of ectopic WNK2 inhibits growth and migration of A172 cells

Considering that our data have so far supported a role for WNK2 as a tumour-suppressor gene in gliomas, we wondered whether restoration of WNK2 expression would inhibit the major cell properties described above. Promoter-methylated A172 cells that lost endogenous WNK2 expression were therefore transfected with a WNK2 cDNA construct. Owing to the inefficiency of transient plasmid transfection into A172 cells, we established pools of stable transfected cells expressing either the WNK2 coding sequence (A172-HW2) or the corresponding empty vector (A172-HEv), and documented the corresponding WNK2 transcript and protein levels (Fig. 7A and 7B). These pools were analysed for their growth and migration properties.

The soft agar colony formation assay demonstrated a significant ($P < 0.05$) decrease in colony numbers formed by A172HW2 cells, in comparison with A172HEv (Fig. 7C).

To examine the effect of WNK2 re-expression on tumour growth *in vivo*, we performed the chick embryo chorioallantoic membrane (CAM) assay (Fig. 7D). The stable A172 cell pools were implanted into the embryo CAM *in ovo* (A172HEv cells, $n = 10$; A172HW2 cells, $n = 15$), and 7 days later the chicken embryos were sacrificed to evaluate tumour growth *ex ovo*, as described (15). The mean perimeter values of the tumours formed by the control A172HEv and the A172HW2 cells were 15405.82 ± 3853.42 μm and 5502.97 ± 2804.83 μm, respectively, being the difference statistically significant ($P < 0.05$) (Fig. 7E). The CAM assay further permits to evaluate angiogenesis modulation. When the number of vessels around the tumours was counted, no statistically significant difference was found between A172HEv and A172HW2 cells (73 ± 10 and 66 ± 17 vessels, respectively) (Fig. 7E).

Furthermore, control A172HEv cells were compared with the stable A172-HW2 cells in wound closure assays. A clear delay in wound closure was observed for A172-HW2 cells due to re-expression of WNK2 (Fig. 8A). Because our previous data found that the loss of WNK2 expression promoted the activation of the small GTPase Rac1, we compared these cells in CRIB (Cdc42/Rac1 interactive binding)-domain pull-down assays. We found lower Rac1 activity in A172HW2 cells than in A172HEv or parental cells (Fig. 8B). In addition, treatment of A172HEv cells with the Rac1 inhibitor NSC23766 also delayed wound closure (data not shown). Together, these data are consistent with a decreased cell growth and migration capacity upon re-expression of WNK2 in promoter-methylated glioblastoma cells, indicating it as a therapeutic target.

DISCUSSION

The main novel findings described in this article are that the loss of WNK2 expression (i) occurs in a subset of 17.5% of adult but not paediatric malignant gliomas, and (ii) leads to increased Rac1 activity and invasion of glioblastoma cells.

Initially, we started by confirming whether epigenetic silencing of the *WNK2* gene through promoter methylation was a

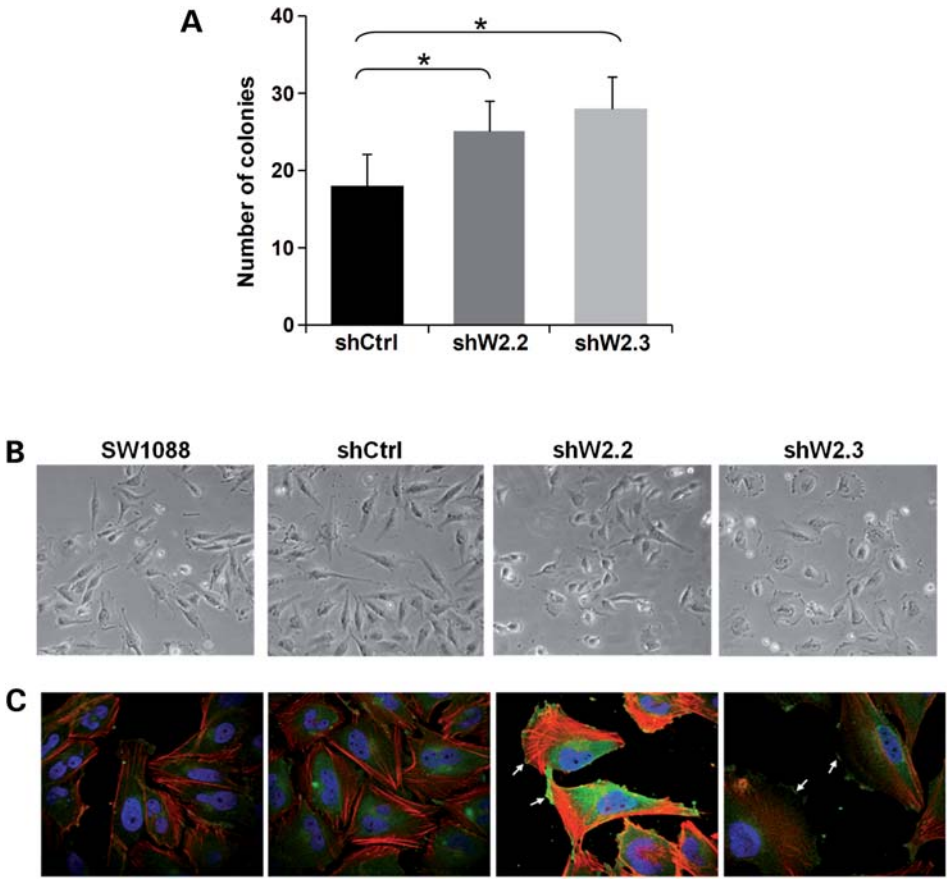


Figure 4. Effect of stable WNK2 depletion in SW1088 cells. (**A**) The tumorigenic growth properties of stable SW1088 clones expressing either shCtrl or shW2.2 or shW2.3 (Fig. 3) were compared in soft agar colony formation assays and the obtained colony numbers graphically displayed. All the experiments were done in triplicate at least three times. Data are represented as the mean ± SD, and differences with a $^{*}P < 0.05$ in the Student's *t*-test were considered statistically significant. (**B**) Changes in cell morphology and actin cytoskeleton in WNK2-depleted SW1088 cells. Bright-field microscopic images showing the morphology of the indicated cell lines. (**C**) Immunofluorescence staining for actin (red) and endogenous Rac1 (green) in the indicated cell lines. Note that cell clones shW2.2 and shW2.3 show increased cell spreading with membrane protrusions which are also detected as Rac1 and actin-containing structures by immunofluorescence.

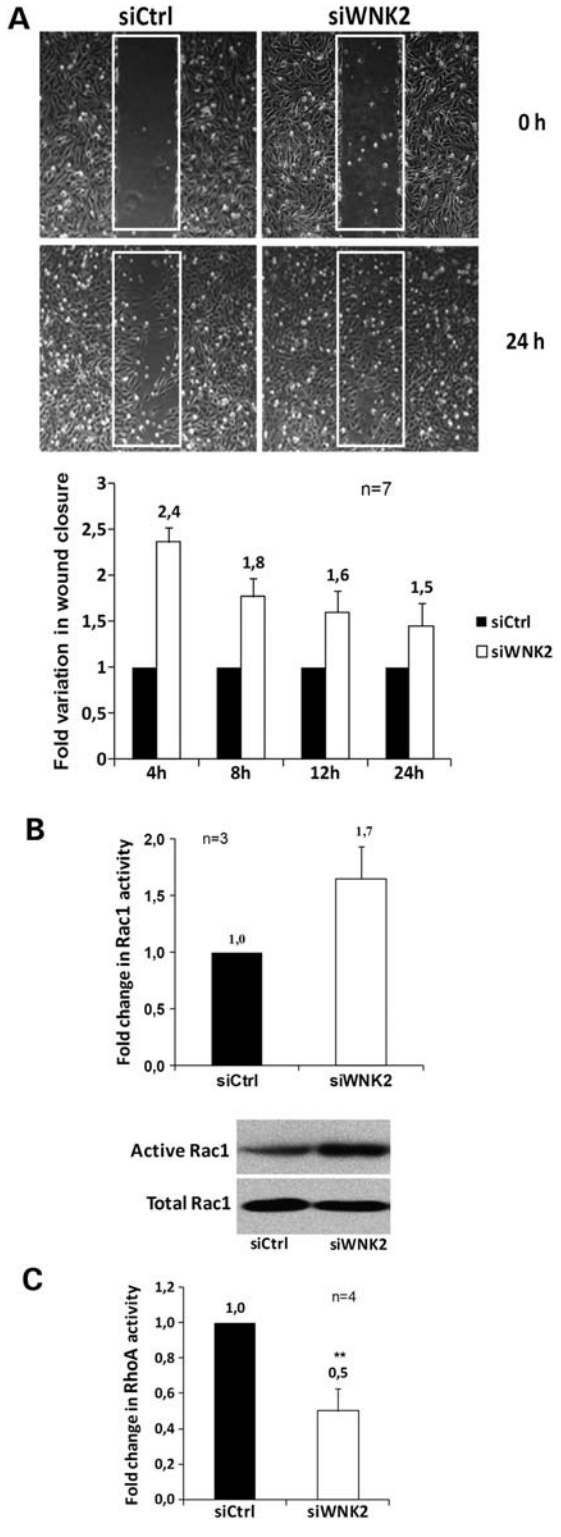
specific target in malignant gliomas. First, our results showed that methylation occurred less frequently in adult gliomas (17.5%; $n = 166$) than in previously described limited series of mainly oligodendrogliomas (94%; $n = 31$) (12) or in meningiomas (83% in grade II, $n = 6$; 71% in grade III, $n = 7$) (13). These differences may in part be overestimated due to the varying sample sizes analysed in the three studies. Nevertheless, our results clearly confirm that *WNK2* gene silencing occurs in gliomas and support the hypothesis of a brain-specific tumour-suppressor gene function (13,16); however, it can formally not be excluded that these WNK2-silenced tumours arose from a glia stem cell type in which the *WNK2* gene is normally methylated.

Second, we found that paediatric malignant gliomas did not share *WNK2* promoter methylation as a common epigenetic event. This underlines our and other authors' reports demonstrating that adult and paediatric gliomas are genetically distinct entities (17–19).

Third, we analysed a presumably alternative WNK2-compromising mutation, the p.A1267T point mutation previously identified by whole-genome sequencing in one glioblastoma sample (14). In 48 of the glioblastoma samples, this mutation could not be detected, indicating that it does not significantly contribute to *WNK2* dysfunction, similar to what has been reported in oligodendroglioma samples (12).

Subsequently, we tested the effect of experimental reduction of WNK2 expression by siRNAs in SW1088 cells, which have no promoter methylation and thus express endogenous WNK2. We found that anchorage-independent cell growth increased under conditions of reduced WNK2 expression and that changes in cell morphology occurred, including increased cell spreading and formation of lamellipodia. Consistently, an increase in Rac1 activation was detected in WNK2-depleted cells, in agreement with the changes in activity that we previously described to occur in HeLa cells (11).

Several recent studies have suggested a predominant role for Rac1 in glioma cell motility and invasiveness, based on the identification of changes in expression or activity of either Rac1 (20–29), or individual Rac-specific guanine nucleotide exchange factors (GEFs) (30–33), or Rac effector proteins (34,35). It is well known that Rac1 drives a mesenchymal type of cell migration, suggesting that this type allows the infiltration of glia-derived tumour cells within the neuronal matrix of the brain (29). Consistent with this role of Rac1, experimental WNK2 suppression in SW1088 cells



led to increased cell migration in wound-healing assays and increased invasion in Matrigel assays. Our findings indicate that distinct mechanisms can contribute to the activation of Rac1 during glioma progression, including the loss of WNK2 expression described in this article. Our previous analysis of WNK2 indicated that its direct target could be a Rho-GEF so that the loss of WNK2 leads to a decrease in RhoA and a reciprocal increase in Rac1 activity (11).

Finally, the tumour-suppressing role of WNK2 in gliomas implies that its re-expression should be able to revert the aggressive phenotype of tumour cells. We clearly found that restoration of WNK2 in the promoter-methylated cell line A172 inhibited colony formation in soft agar and the cell migration capacity, including the endogenous levels of GTP-bound active Rac1. Moreover, we found that tumour growth was reduced using the *in vivo* CAM assay.

Together our data support an important role for WNK2 in the control of glia-derived cell migration and tumorigenic growth and provide mechanistic insights into the pathways involved.

MATERIALS AND METHODS

Patient samples and cell lines

Representative formalin-fixed paraffin-embedded blocks from 227 glioma tissues (166 were from adult and 61 were from paediatric patients) were retrospectively retrieved from pathology archives of the Department of Pathology at three hospitals in northern Portugal—Hospital São João, Porto, Hospital Santo António, Porto, and Hospital Pedro Hispano, Matosinhos, and from the UK at the King's College Hospital, London, and St George's Hospital, London (Supplementary Material, Table S1). The cohort includes astrocytic and oligodendroglial tumours, of diverse malignant grades including 115 adult glioblastoma, which were all classified according to the WHO criteria (1). Follow-up data were available in 133 adult patients (range: 0–64 months, mean: 12.8 ± 1.0 months) and only in 18 paediatric patients (range: 0–80 months, mean: 21.7 ± 5.2 months). The procedures for this study were approved by local and multicenter Ethical Review Committees, and, in accordance with institutional ethical standards, the biological samples were unlinked and unidentified from their donors.

Eight glioblastoma cell lines were used in this study and include SW1088, SW1783, U87-MG and A172 (obtained from ATCC—American Type Culture Collection), SNB-19 and GAMG (obtained from DSMZ—German Collection of Microorganisms and Cell Cultures) and U251 and U373

Figure 5. Effect of transient WNK2 depletion in SW1088 cells. (**A**) Cells were transiently transfected with control (siCtrl) or WNK2-specific (siWNK2) siRNAs under the conditions documented in Figure 3 and analysed. (A) Representative images from wound-healing migration assays, in which scratch wounds were made with a pipette tip across a confluent cell monolayer, and distances between the wound edges measured after 8, 12 and 24 h. In the graph below, the migration distances determined in seven independent assays are expressed as fold differences compared with parental cells and show the distances migrated in relation to time 0. (**B**) Activation levels of endogenous Rac1 as determined under the same transfection conditions by CRIB-domain pull-down assays and analysed by western blot comparing total and GTP-loaded Rac1 fractions ($n = 7$). (**C**) Control or WNK2-depleted SW1088 cells were lysed and Rho A activation levels determined in a G-Lisa assay ($n = 4$). Note the increase in cell migration and Rac1 activation accompanied by a decrease in active RhoA following transient WNK2 depletion in parental SW1088 cells. Statistically significant differences are indicated as $^{*}P < 0.05$ or as $^{*}P < 0.005$.

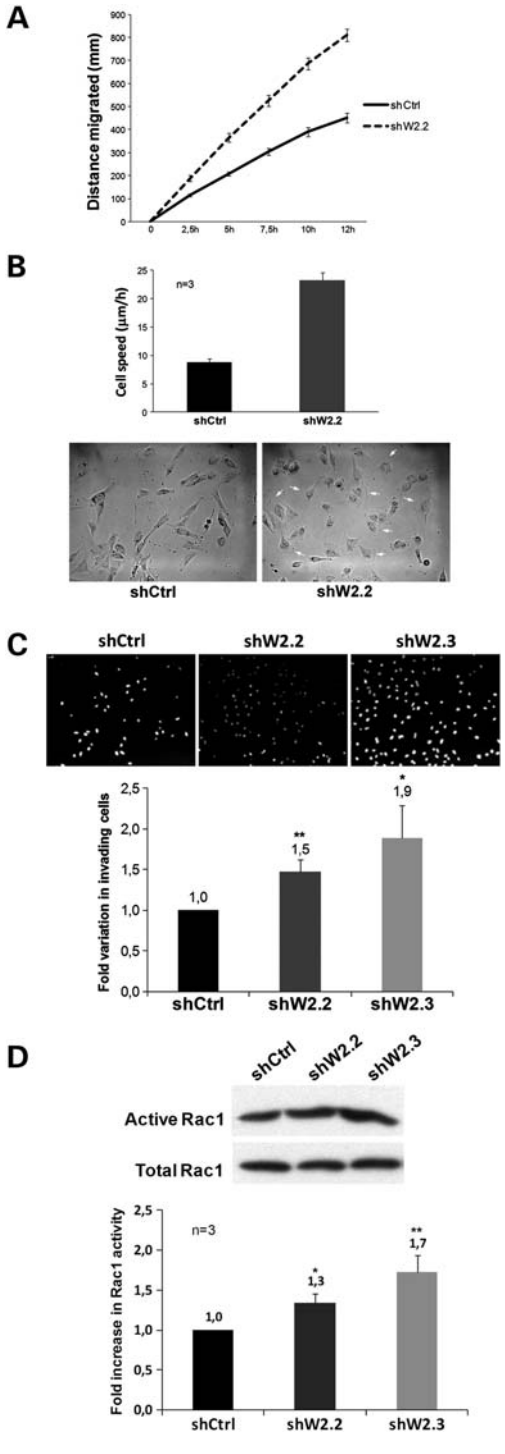


Figure 6. Effect of stable WNK2 depletion in SW1088 cells. (**A**) Cells expressing the indicated shRNA vectors were characterized for their migration capacity in wound closure using *in vivo* time-lapse microscopy (see also Supplementary Material, Movies M1 and M2). Shown is the quantification ($n = 3$) of the migrated distances by comparing the automatically acquired images at the indicated time points. (**B**) Live cells seeded at low density were analysed for their spontaneous motility properties. Shown is the quantification ($n = 3$) of the mean speed (distances migrated during 12 h) of about 85 shCtrl and shW2.2 cells as well as representative still images picked from the Supplementary Material, Movies M3 and M4. Note that WNK2-depleted cells formed extensive lamellipodia (white arrows) and were more motile. (**C**) The invasive properties of the indicated cell lines were determined in Matrigel invasion assays. Shown are representative microscopic images of the amount of cells able to cross the membrane barrier and a graphical summary of six independent experiments. (**D**) Activation levels of endogenous Rac1 determined in lysates from the three stable SW1088 clones by CRIB-domain pull-down assays ($n = 3$). Shown is a representative western blot comparing total and GTP-loaded Rac1 fractions and a graphical quantification obtained from three independent experiments. Statistically significant differences are indicated as $^{*}P < 0.05$ or as $^{*}P < 0.005$.

(obtained from Costello Laboratory and described in ref. 36). Their mutation profile of major cancer-associated genes is shown in Supplementary Material, Table S3. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) medium as previously described (15) and regularly checked for the absence of mycoplasm infection. Authentication of cell lines was performed by IdentiCell Laboratories [Department of Molecular Medicine (MOMA) at Aarhus University Hospital Skejby in Århus, Denmark] in August 2011. Genotyping confirmed the complete identity of all cell lines, with the exception of U373, which was shown to be a subclone of U251 cell line.

MSP, *WNK2* promoter sequencing and mutation analysis

For the isolation of genomic DNA, selected areas from the formalin-fixed paraffin-embedded blocks containing at least 85% of tumour tissue were macro-dissected into a microfuge tube using a sterile needle (Neolus, 25G, 0.5 mm) and DNA isolated using QIAamp® DNA Micro Kit (Qiagen, Hilden, Germany), as previously described (37). DNA from the cell lines was isolated using TRIzol® Reagent (Invitrogen S.A., Barcelona, Spain) as recommended by the manufacturer.

For MSP, 500 ng of DNA were bisulphite-treated using EZ DNA Methylation Golf Kit (Zymo Research Corporation, Irvine, CA, USA), as previously described (37). Specific primers to distinguish methylated (131 bp PCR product 1, Fw: 5′-CGTTCGTTTTGTGAGTGTC; Rv: 5′-ACGACGACTCCACAAAAA) from unmethylated DNA (131 bp PCR product 2; Fw: 5′-GTTTGTTTGTTTTGTGAGTGTT and Rv: 5′-CCAACAACAACTCCACAAAAA) were designed using the Methyl Primer Express Software v1.0. CpGenome Universal Methylated DNA (Chemicon Millipore, Billerica, MA, USA) was used as methylated control and blood DNA of a young healthy individual was used as unmethylated control. Bisulphite sequencing of the *WNK2* promoter covered 73 contiguous CpGs in the *WNK2* CpG island and was analysed by sequencing 10 subclones of PCR products from bisulphite-treated DNA, as previously described (12,13).

The prevalence of the WNK2-A1267T missense mutation was determined following PCR amplification of exon 16 with primers WNK2-A1267T-F (5′-CGA GCA GAT GAA GGA TGT CA) and WNK2-A1267T-R (5′-GAA TGA GGT GGA GGG TCA GA) and direct sequencing of the obtained product.

Cell culture, transfections and RNA interference

A172 and SW1088 cell lines were maintained in DMEM, supplemented with 10% fetal bovine serum (Invitrogen) and

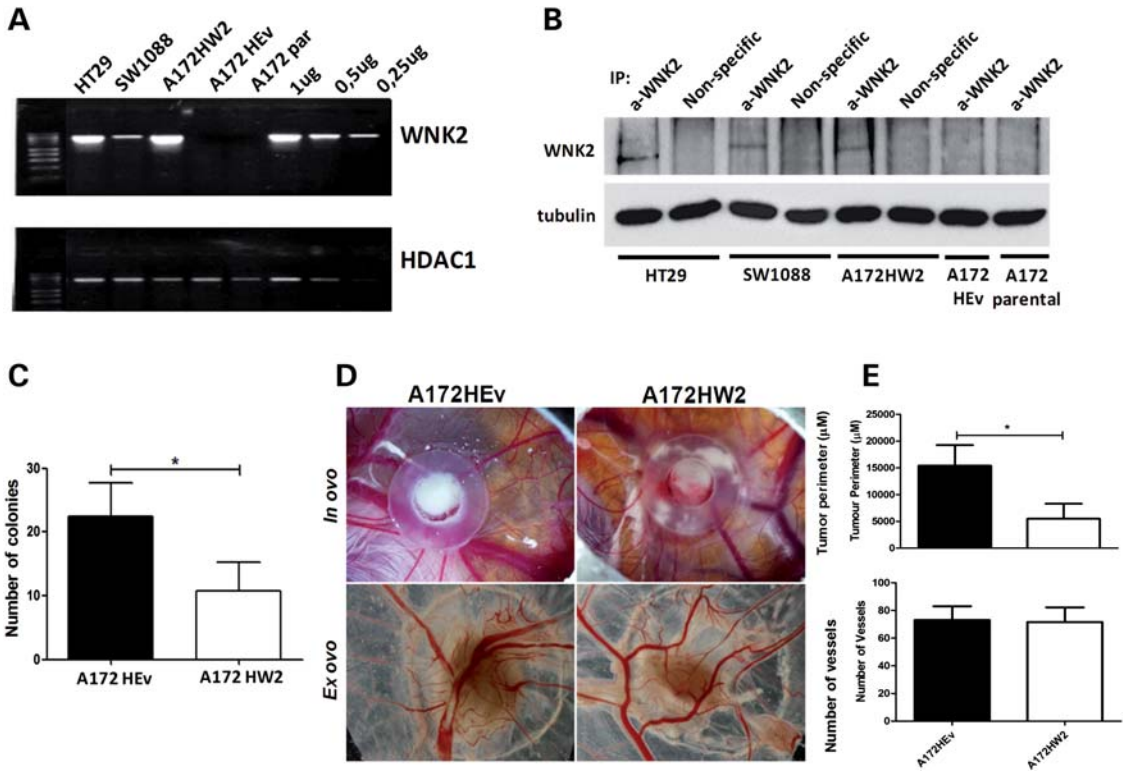


Figure 7. Stable re-expression of WNK2 in A172 cells. (**A**) RT-PCR analysis of WNK2 transcript comparing parental (A172 par) and empty vector (A172 HEv) control cells with WNK2-transfected A172 cells (A172 HW2). Colon HT29 and SW1088 cells were included as positive controls. Two serial dilutions of HT29-input RNA were co-amplified to guarantee semi-quantitative PCR conditions. (**B**) Detection of WNK2 protein in the same cell lines after immunoprecipitation and western blot using a specific anti-WNK2 antibody. Note the re-expression of WNK2 in A172 HW2, but not in parental or empty vector-transfected A172 HEv control cells. (**C**) *In vitro* tumorigenic growth properties of A172 HEv control and WNK2-transfected A172 cells (A172 HW2) were compared in soft agar colony formation assays and the obtained colony numbers graphically displayed. (**D**) *In vivo* tumorigenic growth properties were analysed in CAM assays. Representative pictures (16× magnification) of CAM assays after 7 days of tumour growth *in ovo* and *ex ovo*. We analysed 25 eggs (10 were injected with A172HEv and 15 with A172HW2 cells) and observed statistically significant smaller perimeter (μm) in the tumours formed by A172HW2 cells (upper panel). The counting of the blood vessels *ex ovo* revealed no differences in the number of vessels recruited to the tumours formed by both cell lines (lower panel). (**E**) Graphical representation of the data quantitation obtained from all images, given as the mean ± SD. Differences with a $P < 0.05$ in the Student's *t*-test are marked by an asterisk and were considered statistically significant.

regularly checked for the absence of mycoplasm infection. Cells were transfected using a reverse transfection protocol in which 1×10^6 cells were trypsinized and seeded together with the premixed plasmid DNA/LipofectAMINE 2000 (Invitrogen) complex, according to the manufacturer's instructions. Cells were analysed after 22 h, and transfection efficiencies of a GFP expression vector found to be ∼40%. Total amounts of transfected plasmid DNA were kept constant at 4 μg per 60 mm dish or at 2 μg per 35 mm dish and adjusted with empty vector if required. siRNAs were obtained from Eurofins MWG-Biotech AG (Ebersberg, Germany) and reverse-transfected using 5×10^5 trypsinized cells and siRNA/LipofectAMINE 2000 complexes containing 300 pmol of siRNA per 35 mm dish or 600 pmol per 60 mm dish. siRNA sequences were siWNK2-a (5′-GCU CGA GGA UGC UGA CAU ATT), siWNK2-b (5′-GGA CGC ACC CGA UGA AAU UTT) and as control siGFP (5′-GGC UAC GUC CAG GAG CGC ACC TT). Cells transfected with siRNAs were analysed after 48 h, and transfection efficiencies found to be ∼60% using an FITC-coupled siRNA (Qiagen). The achieved reduction in target gene expression was determined in each experiment by removing a 30 μl of aliquot from the cell lysate for the extraction of total RNA, as described below.

For the selection of stable cell lines with reconstituted WNK2 expression in A172 cells, the WNK2 cDNA was subcloned as an *Eco*RI/*Eco*RV fragment into pcDNA3-Hygro (Invitrogen). After cell transfection (see above), stable cell pools were obtained following treatment with 200 μg/ml of Hygromycin B (Sigma-Aldrich Quimica, Madrid, Spain). A control stable cell pool transfected with the corresponding empty vector was also selected. For the selection of stable SW1088 cell lines with depleted endogenous WNK2 expression, shRNA encoding plasmids [part of the LKO.1 shRNA constructs obtained from the RNAi Consortium (TRC) (Broad Institute, Cambridge, MA, USA)] containing either a non-specific shRNA sequence (shCtrl) or two previously validated shRNAs sequences targeting WNK2 were transfected. Cell clones were obtained following the treatment of transfected cells with 2.5 μg/ml of puromycin (Sigma-Aldrich).

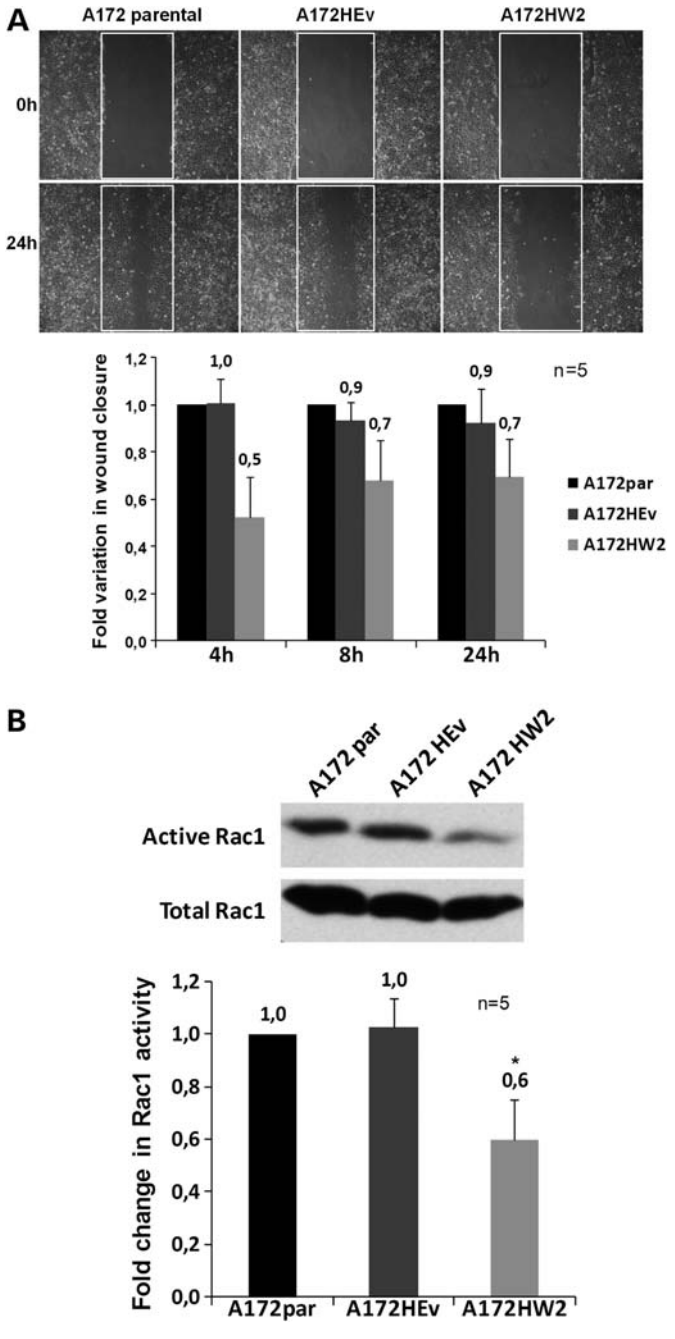


Figure 8. Effect of WNK2 re-expression on A172 cell migration. (**A**) Representative images from wound-healing migration assays (see legend to Fig. 5). In the graph below, the migration distances determined in five independent assays are expressed as fold differences compared with parental cells and show the distances migrated in relation to time 0. (**B**) Activation levels of endogenous Rac1 were determined in the same three cell lines by CRIB-domain pull-down assays ($n = 5$) as described in the legend to Figure 5. Note the decrease in cell migration and Rac1 activation following WNK2 re-expression in A172 HW2 cells. Statistically significant differences are indicated as $^{*}P < 0.05$.

Soft agar colony formation assay

For soft agar colony formation, 1 ml underlayer (base agar layers) consisting of 0.6% agar medium was prepared in six-well plates by combining equal volumes of 1.2% Noble agar with 2× DMEM medium containing 20% FBS. Cells were trypsinized, centrifuged and resuspended in 0.35% agar medium (top agar layer; equal volumes of 0.7% Noble agar and 2× DMEM with 20% FBS) before 5×10^3 cells were plated onto the previously prepared base agar layers. The cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for 3 weeks and the colonies formed stained with 0.05% violet crystal for 15 min. Stained colonies were photographed in a stereomicroscope (Olympus Z2× 16) using a digital camera (Olympus DP71) (Olympus, Hamburg, Germany) and counted with the Image J software. Results are expressed as the mean number of colonies per field. The assay was done in triplicate and repeated at least three times.

Cell lysis and immunoprecipitation, Rac1-CRIB pull-down or Rho G-Lisa assays

Cells in 60 mm dishes were lysed on ice in 250 µl of lysis buffer (20 mM Tris–HCl, pH 7.5, 1% NP-40, 130 mM NaCl, 10% glycerol, 10 mM $MgCl_2$) containing 10 mM NaF, 0.1 mM Na_3VO_4, 1 mM DTT and a protease inhibitor cocktail composed of 1 mM PMSF, 1 mM 1,10-phenanthroline, 1 mM EGTA, 10 mM E64 and 10 µg/ml of each aprotinin, leupeptin and pepstatin A (all from Sigma-Aldrich). For RNA extraction, a 30 µl of aliquot was removed and processed as described below. For protein analysis, a 40 µl of aliquot was added to 10 µl of 5× Laemmli sample buffer, boiled for 10 min, centrifuged at 2500*g* for 30 s and analysed as given below. To document WNK2 protein levels in A172HW2 cells, a confluent 100 mm dish was lysed and immunoprecipitated with 15 µg of a rabbit polyclonal WNK2 antibody, SPT81 (10). For comparison, an equivalent immunoprecipitation was performed in SW1088 and in colorectal HT29 cell lysates as a previously described positive control (11). The Rac1 CRIB-domain pull-down assay and the activation assays for RhoA were performed as described (11,38), the latter using the G-Lisa RhoA luminescence-based Biochem kit (Cytoskeleton, Denver, CO, USA).

Transcript expression analysis and semiquantitative PCR

Total RNA was extracted from cell lines or lysates with the RNAeasy kit (Qiagen) and 1 µg reverse-transcribed using random primers (Invitrogen) and Ready-to-Go You-Prime beads (GE Healthcare, Buckinghamshire, UK). Primers and reaction conditions for specific amplification of WNK2 and for RNA polymerase II (Pol II) were as described (11). These semi-quantitative amplification conditions were experimentally controlled by co-amplification of serial dilutions of a cDNA sample. Products were separated on 1.5% agarose gels and band intensities quantified using the Image J software followed by normalization to Pol II. No amplification was obtained when RNA was mock-transcribed without adding reverse transcriptase.

Antibodies and western blot procedures

Protein samples were separated in a 10% SDS–PAGE Mini-Protean III gels (Bio-Rad, Hercules, CA, USA). Proteins were transferred onto a PVDF membrane (Bio-Rad) using

a Mini Trans-Blot cell (Bio-Rad) at 100 v for 60 min and Coomassie-stained to check for equal transfer. Membranes were blocked in TBS, 0.1% Triton X-100, 5% milk powder, probed using the indicated antibodies, then incubated with a secondary peroxidase-conjugated antibody (Bio-Rad) and specific binding detected in a chemiluminescence reaction. For densitometric estimation of protein quantities, the luminescence film exposures from at least three independent experiments were digitalized and analysed using the ImageJ software (NIH). The antibodies used were anti-α-tubulin (clone B5-1-2) from Sigma-Aldrich, monoclonal anti-Rac1 (clone 23A8) from Upstate Millipore, Billerica, MA, USA, and rabbit anti-WNK2 (10).

Immunofluorescence microscopy

Parental and selected shRNA-expressing SW1088 cells were grown on cover slips and fixed with 3.7% paraformaldehyde in PBS followed by permeabilization with 0.1% Triton X-100 in PBS. Cells were washed 3 × 5 min in PBS/0.01% Tween 20 (PBS-T), then labelled for 1 h with primary anti-Rac1 antibody, washed 3 × 5 min in PBS-T and incubated again for 1 h with a mixture of secondary anti-mouse Alexa-488 antibody (Invitrogen-Molecular Probes) and 0.2 μg/ml phalloidin-TRITC (Sigma-Aldrich). Cover slips were washed again 3 × 5 min in PBS-T, post-fixed for 15 min in 4% (v/v) formaldehyde and mounted on microscope slides with Vectashield (Vector Laboratories, Burlingame, CA, USA). Digital images were recorded on a Leica TCS SPE (Leica Microsystems, Wetzlar, Germany) confocal microscope and processed with the Adobe Photoshop software.

Cell migration and invasion assays

For wound-healing assays, either transfected cells or stable cell lines were allowed to grow to a confluent monolayer in 6-well or 12-well plates before a wound was carefully made with a pipette tip so that the neighbouring cells were only minimally disturbed. The medium was replaced by fresh complete medium and wound closure monitored by time-lapse photography for 12 h, using a phase contrast microscope and a 10× magnification. Migration was measured on digital images by determining the mean remaining distance between the wound edges. In some experiments, cells were treated with 200 μM of the inhibitor NSC23766 (Calbiochem) for 16 h prior to wounding.

Matrigel invasion assays were performed using 8 μm-pore size BD BioCoat™ Matrigel Invasion Chambers (BD Biosciences, San Jose, CA, USA). The upper compartment of the chamber received 2.5×10^4 cells, whereas the lower compartment contained only fresh medium supplemented with 10% FBS. After 24 h incubation at 37°C, the upper surface of the filter was cleared from residual cells with a cotton swab, the filter washed with PBS, then fixed with cold methanol and invasive cells attached to the lower filter surface stained with DAPI. Images were recorded on a Leica SPE confocal microscope at 10× magnification and invasive cells counted.

Time-lapse microscopy for cell motility and wound-healing

For motility and migration assays of shRNA-transfected clones, cells were either seeded in 6-well or 12-well dishes and left to adhere for 24 h at 37°C in a temperature- and 5% CO_2-controlled micro-chamber module, as described (39). For each time-lapse experiment, bright-field images were acquired in intervals of 5 min during 12 h using a 20× objective of an Axiovert 200M microscope and the AxioVision software (Carl Zeiss, Jena, Germany). For the wound-healing assays, distance measurements were done between the wound edges at several time points. For the cell motility assay, the positions of 80–95 individual cells seeded at low density were marked (based on the centre of their nuclei) for each experimental condition and followed in the sequential image series. The moved distances were determined manually and expressed as cell speed (μm/h).

Chick CAM assay

To assess *in vivo* tumour proliferation and angiogenesis, we used the CAM assay as previously described (15,40). Briefly, fertilized chicken eggs were incubated at 37°C and 70% humidity, and on day 4 of development, a window was made into the shell, which was sealed with tape, and the eggs were returned to the incubator. On day 9 of development, small plastic rings were placed on the CAM, and on day 10 of development, 3×10^6 cells, resuspended in 20 μl of DMEM medium, were injected in the rings over the CAM. On day 17 of development, the tumour formed was photographed *in ovo* using a stereomicroscope (Olympus S2 ×16). The chicken embryos were sacrificed at −80°C for 10 min, and the CAM and tumours were fixed with formaldehyde at 4% and photographed *ex ovo*. The perimeter of the tumours was measured using the Cell B software (Olympus), and blood vessels were manually counted.

Statistical analysis

Correlations between *WNK2* methylation, expression and clinical data of the patients were performed using the chi-square test. Cumulative survival probabilities were calculated using the Kaplan–Meier method. Differences between survival rates were tested using the log-rank test. The statistical analysis was performed using the SPSS software for Windows, version 17.0. For *in vitro* assays, simple comparison between two different conditions were analysed using Student's *t*-test. The level of significance in the statistical analyses is indicated as $^*P < 0.05$ or as $^{**}P < 0.005$.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by the Portuguese Fundação para a Ciência e Tecnologia (grant PTDC/SAU-OBD/100079/2008

to P.J., grants PTDC/SAU-IMU/110303/2009 and PTDC/SAU-MII/100780/2008 to L.F.M. and PEst-OE/BIA/UI4046/2011 to the BioFig research unit; fellowships SFRH/BD/36463/2007 to O.M. and UMINHO/BI/016/2011 to F.P.). L.F.M. receives support from Fundação Luso-Americana para o Desenvolvimento and C.J. acknowledges NHS funding to the Biomedical Research Centre.

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PUBLICATIONS

Paper 12

Journal of Pathology
J Pathol 2013
Published online in Wiley Online Library
(wileyonlinelibrary.com) DOI: 10.1002/path.4143

ORIGINAL PAPER

P-cadherin functional role is dependent on E-cadherin cellular context: a proof of concept using the breast cancer model

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Abstract

P-cadherin overexpression is associated with worse breast cancer survival, being a poor prognostic marker as well as a putative therapeutic target for the aggressive triple-negative and basal-like carcinomas (TNBCs). Previously, we have shown that P-cadherin promotes breast cancer invasion of cells where membrane E-cadherin was maintained; however, it suppresses invasion in models without endogenous cadherins, like melanomas. Here, we investigated if P-cadherin expression would interfere with the normal adhesion complex and which were the cellular/molecular consequences, constituting, in this way, a new mechanism by which E-cadherin invasive-suppressor function was disrupted. Using breast TNBC models, we demonstrated, for the first time, that P-cadherin co-localizes with E-cadherin, promoting cell invasion due to the disruption caused in the interaction between E-cadherin and cytoplasmic catenins. P-cadherin also induces cell migration and survival, modifying the expression profile of cells expressing wild-type E-cadherin and contributing to alter their cellular behaviour. Additionally, E- and P-cadherin co-expressing cells significantly enhanced *in vivo* tumour growth, compared with cells expressing only E- or only P-cadherin. Finally, we still found that co-expression of both molecules was significantly correlated with high-grade breast carcinomas, biologically aggressive, and with poor patient survival, being a strong prognostic factor in this disease. Our results show a role for E- and P-cadherin co-expression in breast cancer progression and highlight the potential benefit of targeting P-cadherin in the aggressive tumours expressing high levels of this protein.

Keywords: P-cadherin; E-cadherin; invasion; motility; breast cancer

Received 24 April 2012; Revised 17 October 2012; Accepted 8 November 2012

Conflict of interest statement: OS has options in the company Olink Biosciences that commercializes the PLA technology.

Introduction

E-cadherin (epithelial cadherin, *CDH1*) is a transmembrane glycoprotein responsible for cell–cell adhesion in epithelial tissues, being one of the most studied invasion suppressor proteins in cancer [1,2]. E-cadherin loss-of-function occurs during cancer progression [3] and is associated with tumours with an infiltrative pattern of growth, such as diffuse gastric and lobular breast cancers [4–7]. Somatic *CDH1* mutations, as well as loss of heterozygosity, promoter hypermethylation or overexpression of transcriptional repressors, have been described as molecular mechanisms restraining E-cadherin normal function in invasive carcinomas [8–10]. Loss or delocalization of both catenins (p120ctn and βctn) from the membrane adhesion complex is usually related to an invasive cancer phenotype, due to cadherin destabilization and disorganization of the actin cytoskeleton [11,12].

However, some invasive epithelial tumours, namely the local advanced inflammatory breast cancer, and some highly metastatic breast cancer cells, such as the 4T1 cell model, maintain normal membrane E-cadherin expression. Interestingly, these cells and tumours show aberrant concomitant expression of another epithelial cadherin, named P-cadherin (placental cadherin, *CDH3*) [13].

P-cadherin is overexpressed in several solid tumours, including breast cancer [14], being expressed in 30% of all invasive carcinomas. It is associated with poor

patient survival and is overexpressed in triple-negative basal-like carcinomas (TNBCs), which still do not have a targeted therapy [13,15–18]. We showed that one of the mechanisms underlying the invasive capacity of P-cadherin overexpression in breast cancer cells is mediated by the secretion of MMPs, which cleave its extracellular domain, producing a P-cadherin soluble fragment with pro-invasive activity [19]. However, while performing an extensive revision of the literature, we noticed that the invasive phenotype mediated by P-cadherin was seemingly dependent on the concomitant expression of E-cadherin: in cell models where P-cadherin showed an invasion promoter function, E-cadherin was always also expressed [20–23]; contrarily, in models where P-cadherin was expressed alone, it was described as an invasion suppressor [24–26] (Supplementary Table 1).

Considering these observations, we evaluated if P- and E-cadherin co-expression could induce an aggressive biological cell behaviour compared to cells just expressing one of each cadherin. Indeed, we found that P-cadherin expression disrupts the normal invasive suppressor function of E-cadherin by decreasing the interaction between E-cadherin and intracellular catenins. We showed that cells co-expressing P- and E-cadherin have a specific molecular signature, as well as an increased invasive and tumourigenic potential. The present work still reinforces the importance of P-cadherin as a prognostic factor in breast cancer and suggests that its overexpression is an alternative mechanism for cancer progression and invasion in E-cadherin-positive breast carcinomas. Therapeutically, this knowledge supports the development of anti-P-cadherin strategies to control highly aggressive breast carcinomas co-expressing both cadherins.

Materials and methods

Cell culture and siRNA transfection

Human breast cancer cell lines were obtained as described: BT20 (ATCC, Manassas, VA, USA) and SUM149 (Dr Stephen Ethier, University of Michigan, MI, USA). Cells were routinely maintained at 37°C, 5% CO_2, in the following media (Invitrogen Ltd, Paisley, UK): DMEM (BT20) and DMEM/HamF12 (1 : 1) (SUM149), supplemented with 10% heat-inactivated fetal bovine serum (Greiner bio-one, Wemmel, Belgium), 100 IU/ml penicillin and, 100 µg/ml streptomycin (Invitrogen). SUM149 medium was supplemented with 5 µg/ml insulin and 1 µg/ml hydrocortisone (Sigma-Aldrich, St Louis, MO, USA).

Transient transfections with small interfering RNAs (siRNAs), specific for P-cadherin and/or E-cadherin silencing (50 nM Hs_CDH3_6 and 100 nM Hs_CDH1_13, respectively; GW Validated siRNA; Qiagen, Valencia, CA, USA), were performed with Lipofectamine™ 2000 (Invitrogen), according to the manufacturer's recommendations. A negative siRNA control, with no homology to any gene, was also used (Qiagen).

Antibodies and chemicals

The following primary antibodies were used: P-cadherin (western blot: mouse, clone 56; BD Biosciences, Lexington, KY, USA; immunofluorescence and PLA: rabbit; Cell Signaling, Boston, MA, USA), E-cadherin (western blot: mouse, clone HECD1; Takara Bio Inc, Shiga, Japan; PLA: rabbit; Cell Signaling), p120ctn and βctn (mouse, clone 98 and clone 14; BD Biosciences), and β-actin (goat, clone I19; Santa Cruz Biotechnologies, CA, USA).

The following chemicals were used: ZVAD.fmk (pretreatment with 20 µM for 2 h; Peptide Institute, Inc, Osaka, Japan).

Immunofluorescence and *in situ* proximity ligation assay (PLA)

Immunofluorescence was performed as previously described [19].

For the PLA, tumour sections were prepared and treated with the same protocol used for immunohistochemistry (IHC). Culture cell lines were deposited on glass slides and fixed with methanol. PLA was performed using the Duolink kit (Olink Bioscience, Uppsala, Sweden) according to the manufacturer's recommendations. Slides were analysed with fluorescence microscopy (Zeiss Imager Z1 microscope) and the Blobfinder V3.2 free software (Centre for Image Analysis, Uppsala, Sweden) was used to quantify the number of blobs (or dots). A detailed description may be found in the Supplementary materials and methods.

Wound-healing assay and BrdU proliferation assay

Wound-healing migration and BrdU proliferation assays were performed as previously described [19]. For the migration assay, 24 h after transfection, cells were replated and left another 24 h to reach confluence. A wound was made and cell migration was evaluated for 24 h. For the proliferation assay, 48 h after transfection, cells were incubated for 6 h with BrdU and fixed with 4% formaldehyde. A detailed description may be found in the Supplementary materials and methods.

Western blot and immunoprecipitation

For immunoprecipitation, 500 µg of protein was incubated with 2 µg of the appropriate antibody. Western blot was performed as previously described [27]. Representative blots were selected to be shown.

Matrigel invasion assay, slow aggregation assay, and TUNEL assay

The Matrigel invasion assay and slow aggregation assay were performed as previously described [19,21].

The TUNEL assay was carried out according to the manufacturer's recommendations (TUNEL kit, Roche). A detailed description may be found in the Supplementary materials and methods.

cDNA microarrays

Total RNA from BT20 cell lines was extracted with the RNeasy Extraction Kit (Qiagen) and quality was assessed by BioAnalyser (Agilent Technologies, Santa Clara, CA, USA). Samples were hybridized onto Agilent 44K microarrays with probes for the Human Genome (Agilent HPAG4112F), following the manufacturer's recommendations, using the Agilent One-Color Microarray-Based Gene Expression Analysis Protocol Version 5.7 (Quick Amp Labeling Kit).

Microarray images were obtained and fluorescence intensity was measured using the Agilent Feature Extraction Software (Version 10.5.1.1) and signal processing was performed according to the Agilent recommendations (GE1-v5_95_Feb07 Protocol). The processed signal was annotated and filtered using BRB Array Tools 3.8.0 (http://linus.nci.nih.gov/BRB-ArrayTools.html). Differences in gene expression were assessed using the Student's t-test implemented in BRB-tools, with a p-value cut-off of 0.01. Only genes where expression was altered by at least 2-fold in relation to the control cell line ($Ecad^{+}Pcad^{+}$) were indicated as differentially expressed.

A hierarchical clustering method was applied to identify groups of differentially expressed genes (DEGs) between samples, and the unsupervised analysis was visualized using the TIGR MeV 4.4.1 program (http://www.tm4.org/mev/). DAVID software was used to analyse gene ontology and pathway enrichment (http://david.abcc.ncifcrf.gov/) [28]. A detailed description may be found in the Supplementary materials and methods.

The complete array data can be viewed in the ArrayExpress microarray database (accession No E-MEXP-3329).

Real-time quantitative RT-PCR (qRT-PCR)

Selected genes were analysed by qRT-PCR using gene-specific TaqMan probes (Applied Biosystems, Foster City, CA, USA). Analysis was performed with the ABI PRISM 7700 Sequence Detection System Instrument and software (Applied Biosystems), following the manufacturer's recommendations. A detailed description of the probes may be found in the Supplementary materials and methods.

GTPase activity assays

GTP-bound RhoA and Rac1/2/3 were measured in protein lysates with a commercially available activation kit (G-LISA^{TM}, Kit #BK124 and #BK125; Cytoskeleton, Inc, Denver, CO, USA), according to the manufacturer's instructions. 30 µg of protein was analysed and activation signals are expressed as fold increase/decrease of control values.

Animal studies

Animal experiments were carried out in accordance with the European Guidelines for the Care and Use of Laboratory Animals, Directive 2010/63/UE. Female N:NIH(s)II:nu/nu nude mice, aged 6–8 weeks, were orthotopically inoculated with 10^6 viable cells in the mammary fat pad using four different BT20 cell lines. Mice were weighed and tumour volume was estimated. Mice were euthanized 35 days after tumour cell inoculation, and primary tumours were extracted, fixed in 10% buffered formalin, and then embedded in paraffin, sectioned, and stained with haematoxylin and eosin. A detailed description may be found in the Supplementary materials and methods.

Tissue samples

A series of 467 primary invasive breast carcinomas, diagnosed between 1978 and 1992, were retrieved from the Pathology Department, Hospital Xeral-Cíes, Vigo, Spain. Patient follow-up information was available for 455 cases. The tumours have been characterized for clinical and pathological features (data are summarized in Supplementary Table 2). The study was conducted under the national regulative law for the handling of biological specimens from tumour banks, being the samples exclusively available for research purposes in retrospective studies.

Immunohistochemistry (IHC)

IHC was performed as previously described [17]. The assessment of immunohistochemical results was based on a semi-quantitative evaluation, as previously reported [15,29]. A detailed description may be found in the Supplementary materials and methods.

Statistical analysis

Statistical analyses of the IHC results were performed by SPSS 15.0 software package for Windows (SPSS, Inc, USA). p values less than 0.05 were considered statistically significant. Concerning the functional *in vitro* assays, data are expressed as mean values of at least three independent experiments ± SD. Student's t-tests were used to determine statistically significant differences ($p < 0.05$). A more detailed description may be found in the Supplementary materials and methods.

Results

E- and P-cadherin co-expression in breast cancer: effects on cell–cell adhesion, invasion capacity, and stabilization of the cadherin/catenins complex.

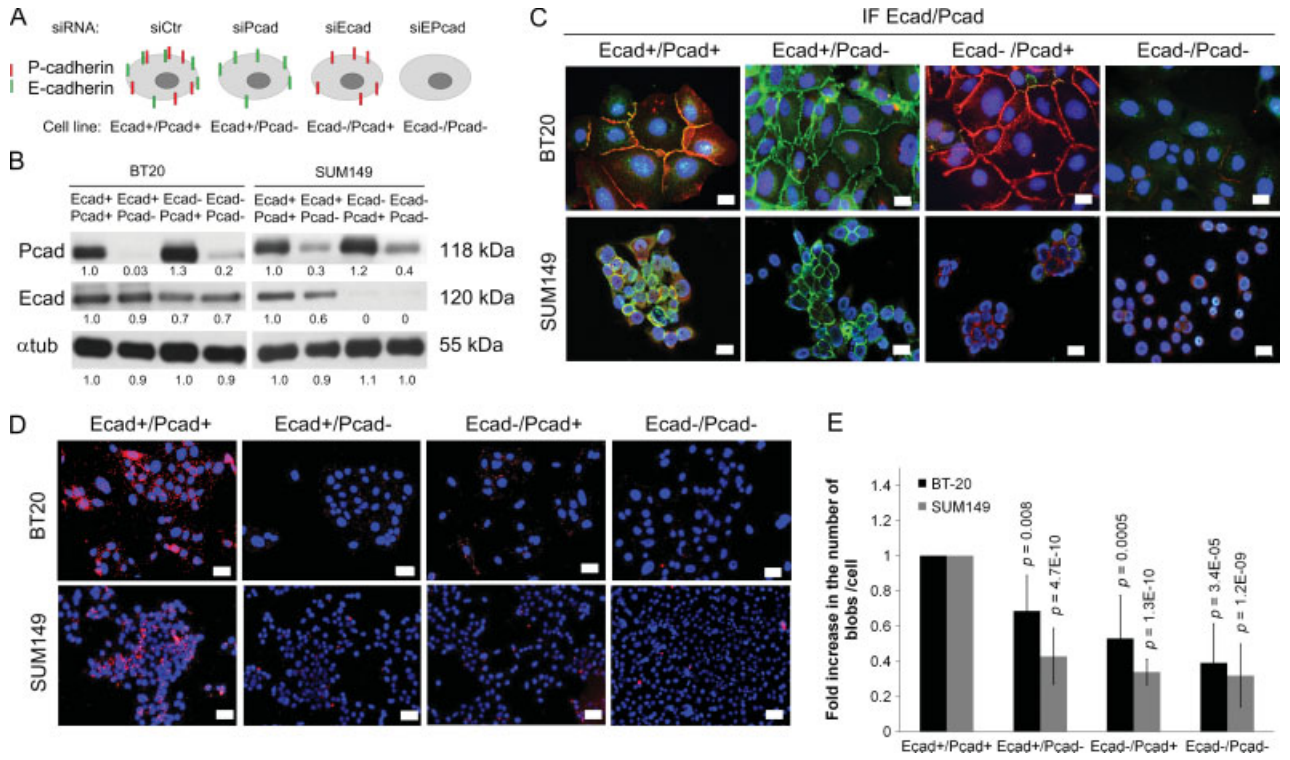

Figure 1. Establishment of a breast cancer cell model where the transcription of E-cadherin or P-cadherin or both cadherins was silenced. (A) Scheme from the cell lines generated after transient siRNA KD for E- and P-cadherin in an initial cell line E- and P-cadherin positive (Ecad+/Pcad+). (B) Analysis of E-cadherin and P-cadherin proteins by western blot in BT20 and SUM149 cells 48 h after specific E- and P-cadherin siRNA transfection. Protein levels of α-tubulin were analysed and used as the loading control. (C) Immunofluorescence for E- and P-cadherin in BT20 and SUM149 cells. (D, E) *In situ* PLA for E- and P-cadherin in BT20 and SUM149 cells and the respective quantification of the number of blobs per cell from three independent experiments. Scale bar = 20 μm.

In order to study the functional effects of P- and E-cadherin co-expression, we established cell models where the expression of both cadherins could be manipulated. BT20 and SUM149 TNBC cell lines were selected, due to high levels of E- and P-cadherin expression. To knock-down (KD) selectively the expression of E- and/or P-cadherin, we used small-interfering RNAs (siRNAs), targeting each one of the two cadherins (Figure 1A). An efficient and specific KD for both cadherins was obtained for at least 168 h after transfection (Figures 1B and 1C). *In situ* proximity ligation assay (PLA) [30], to detect E- and P-cadherin proximity, produced positive signals in both parental cell lines, showing a putative interaction between proteins (Figure 1D). As expected, decreased E- and P-cadherin proximity was detected in all cell lines, where one or both cadherins were silenced in comparison with the control cells (Figures 1D and 1E).

To evaluate the functional effects on cell–cell adhesion, a non-quantitative aggregation assay was performed. The parental $Ecad^+/Pcad^+$ cells formed small aggregates on the top of soft agar (Figure 2A and Supplementary Figure 1A), as did the E-cadherin ($Ecad^+/Pcad^-$) or P-cadherin ($Ecad^-/Pcad^+$) positive cells, where cell aggregation was apparently maintained or even increased. Conversely, when both cadherins were silenced, the cells did not aggregate (Figure 2A and Supplementary Figure 1A).

Concerning cancer cell invasion, $Ecad^+/Pcad^+$ co-expressing cells or $Ecad^-/Pcad^-$ cells were significantly more invasive than the $Ecad^+/Pcad^-$ or $Ecad^-/Pcad^+$ cells ($p < 0.01$) (Figure 2B), showing that invasion can be due to either lack of expression or the co-expression of both cadherins.

In situ PLA was performed to measure the proximity between both cadherins and cytoplasmic catenins (p120ctn and βctn), which are important for the stabilization of the adhesion complex at the cell membrane. The independent silencing of each cadherin promoted a significant increase in interaction at the cell membrane between the cadherin that remained expressed and both catenins (Figure 2C and Supplementary Figure 2A), compared with the interaction detected in $Ecad^+/Pcad^+$ cells. As expected, in $Ecad^-/Pcad^-$ cells, the interaction with both catenins was lost (Figures 2C and 2D). These results were further confirmed by immunoprecipitation (Supplementary Figure 2B). Curiously, catenins maintained their membrane expression in $Ecad^+/Pcad^+$ cells, as well as in cells expressing only one cadherin (E- or P-cadherin) (Supplementary Figure 2C).

E- and P-cadherin co-expression in breast cancer cell migration and cell death

E-cadherin loss of expression/function also results in increased cell migration and survival [31–33]. Taking

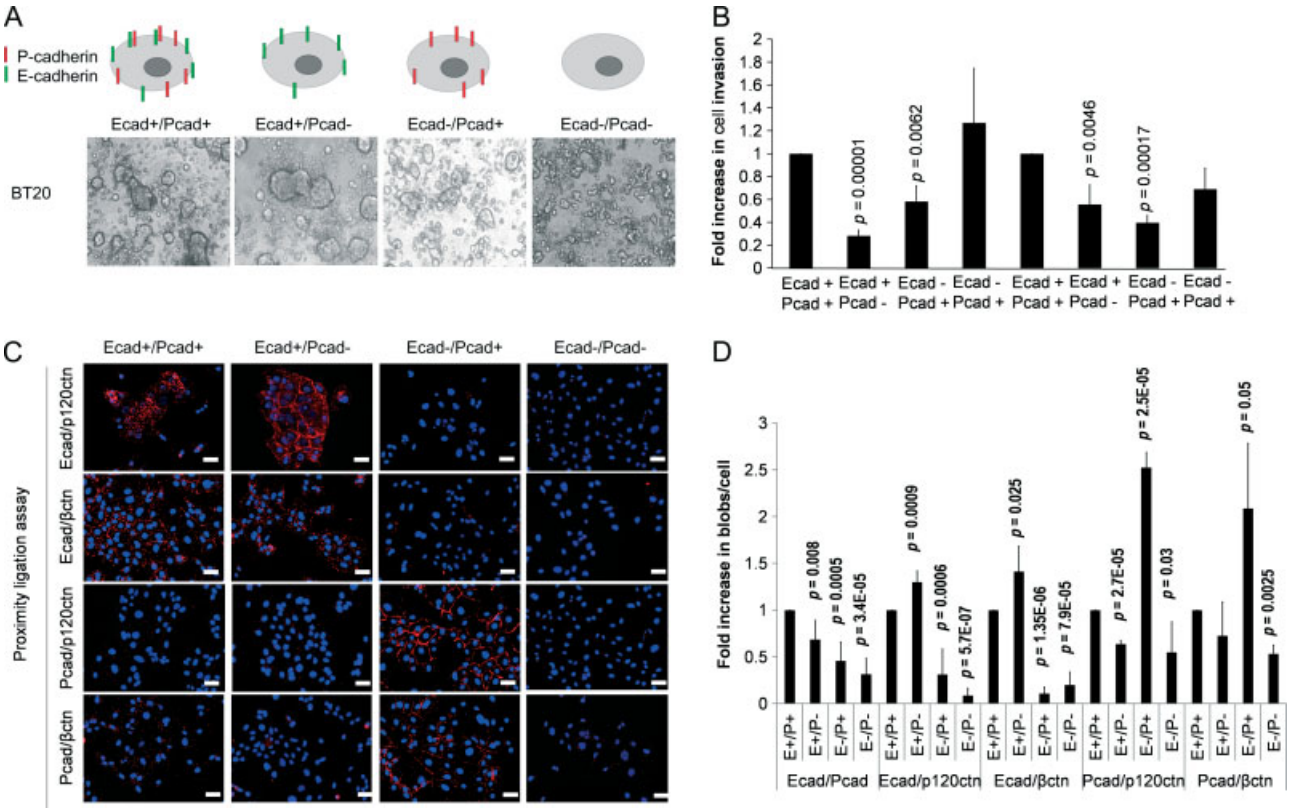

Figure 2. Functional evaluation of E- and P-cadherin expression in breast cancer cells. AA) Cell–cell adhesion was evaluated by the slow aggregation assay in the BT20 cell line. (B) Matrigel invasion assay for the BT20 and SUM149 cell lines after the knockdown of E- and P-cadherin transcription. At least three independent experiments were performed. *p* values are relative to the control cell line and indicate statistically significant results. (C, D) *In situ* PLA using E-cadherin, P-cadherin, p120ctn, and βctn specific antibodies in BT20 cells and the respective quantification of the number of blobs per cell from three independent experiments. Scale bar = 20 μm.

this into consideration, we investigated if there were alterations in these two *in vitro* cellular behaviours, using the breast cancer models established in this study.

The wound healing assay showed that $Ecad^+/Pcad^+$ cells are more motile than $Ecad^+/Pcad^-$ cells ($p < 0.001$) (Figures 3A and 3B and Supplementary Figure 1B). Contrarily, $Ecad^-/Pcad^+$ cells showed a significant increase in cell migration ($p < 0.001$); importantly, this result was not due to an increase in the cell proliferation rate of P-cadherin-expressing cells (Supplementary Figure 3). Unexpectedly, KD of both cadherins resulted in a decrease in cell migration for BT20 cells (Figures 3A and 3B and Supplementary Figure 1B). Although $Ecad^-/Pcad^-$ cells migrated faster than $Ecad^+/Pcad^-$ cells, this difference was not statistically significant.

The functional role of E- and P-cadherin in cell survival was also analysed by the exposure of cells to an apoptotic stimulus. Only $Ecad^+/Pcad^-$ cells showed a significant increase in the percentage of cell death ($p < 0.0001$, Figure 3C and Supplementary Figure 1C). This effect was minimized when cells were treated with a pan-inhibitor of caspases (ZVAD); however, the levels of cell death in $Ecad^+/Pcad^-$ cells, even after ZVAD treatment, were still significantly higher compared with control cells ($p < 0.05$, Figure 3C). Down-regulation of E-cadherin ($Ecad^-/Pcad^+$) resulted in a significantly decreased level of cell death for the SUM149 cell line ($p = 0.0005$, Supplementary Figure 1C), although the effect in BT20 cells was not as prominent ($p = 0.07$, Figure 3C). Therefore, P-cadherin expression and/or loss of E-cadherin expression induce a selective advantage to cancer cells by promoting survival in apoptotic conditions.

Gene expression profile of E- and P-cadherin co-expressing breast cancer cells

The above results prompted us to determine the influence of E- and P-cadherin expression in gene expression deregulation. After E- and P-cadherin KD, mRNA profiles were determined for BT20 cells. The profiles identified 382 genes, whose expression was deregulated (DEGs) ($p < 0.01$; > 2-fold increase/decrease), in at least one of the four groups in relation to the control cells ($Ecad^+/Pcad^+$). The complete array data can be viewed in the ArrayExpress microarray database (accession No E-MEXP-3329).

As expected, *CDH1* and *CDH3* genes were significantly altered after silencing (0.1- and 0.2-fold expression, respectively) compared with control cells. 32 DEGs were common to the three conditions tested, when compared with cells expressing both cadherins, and 15 DEGs were shared by cells with KD of one of the cadherins. Interestingly 28 and 58 DEGs were

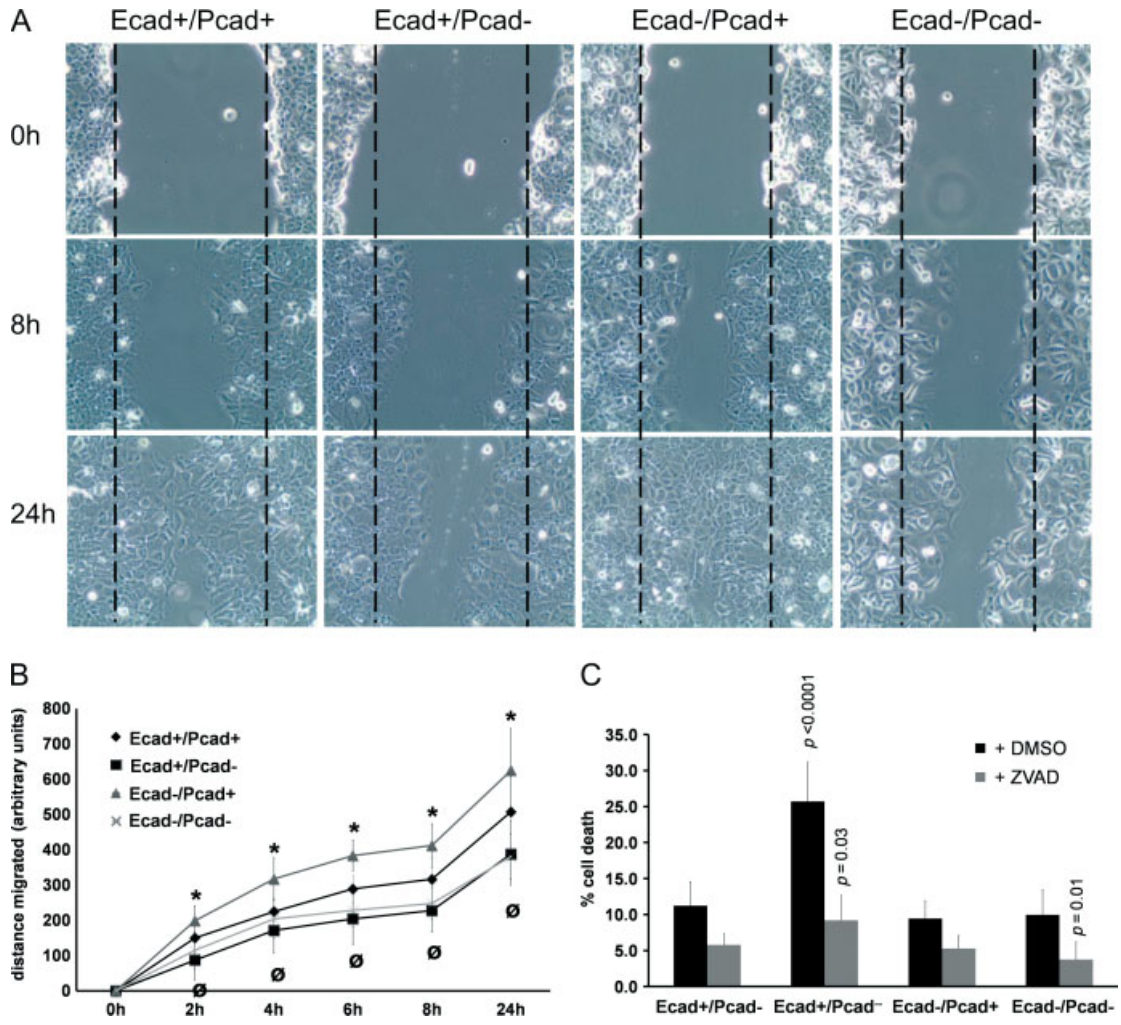

Figure 3. Cell migration and survival in E- and P-cadherin co-expressing breast cancer cells. (A) Representative experiment from a wound healing migration assay in BT20 cells. (B) Quantification of the wound healing migration assay for BT20 cells from three independent experiments performed in duplicate. Significantly increased cell migration between Ecad−/Pcad + cells compared with the Ecad+/Pcad + cell line was observed (represented as *; 2 h – $p = 0.0002$; 4 h – $p = 0.001$; 6, 8, and 24 h – $p \leq 0.0001$). Significantly decreased cell migration between Ecad+/Pcad − cells compared with the Ecad+/Pcad + cell line was observed (represented as ø; 2 h – $p = 0.0007$; 4, 6, and 8 h – $p \leq 0.0001$; 24 h – $p = 0.0006$). (C) TUNEL assay for BT20 cells after pretreatment with DMSO or 20 μM ZVAD. Statistically significant p values are indicated.

specific to P-cadherin or E-cadherin silencing, respectively (Figure 4A).

The transcriptional gene expression profile showed that distinct signalling pathways were differentially modified by both cadherins (Figure 4B). Analysis of gene ontology identified groups of genes significantly enriched in each condition. Both cadherins were involved in GTPase-mediated signal transduction and actin cytoskeleton organization, which is consistent with their role in cell adhesion, invasion, and migration. Moreover, apoptosis signalling was specifically deregulated after P-cadherin silencing (Table 1).

qRT-PCR analysis was performed to validate some of the DEGs found as differentially expressed by microarrays in the different BT20 cells (Figure 4C), namely *ACTG1*, *RAC1*, *IFI6*, *TAX1BP3*, and *UNC 13B*. To see how general the microarray results were, these same genes were also tested in SUM149 cells. Despite the down-regulation of the *CDH1* and *CDH3* mRNAs in silenced cells, the same differential expression of the majority of the genes was also found (Figure 4C and Supplementary Figure 4).

Since consistent results were found for *ACTG1* and *RAC1* mRNA expression in both cell models, we studied the activity of Rho/Rac GTPases, since they play important roles in actin cytoskeleton organization, cell adhesion, migration, and malignant transformation [34]. Additionally, a link between cadherin unbounded p120ctn and activation of Rac1 and inhibition of RhoA GTPases has also already been shown [35]. In fact, we found that $Ecad^{+}/Pcad^{+}$ cells are the ones with increased Rac activity; however, no significant differences were found concerning RhoA activation (Supplementary Figure 5).

Effect of E- and P-cadherin co-expression on tumour growth in nude mice

In order to investigate the effect of E- and P-cadherin co-expression on *in vivo* tumour growth, we

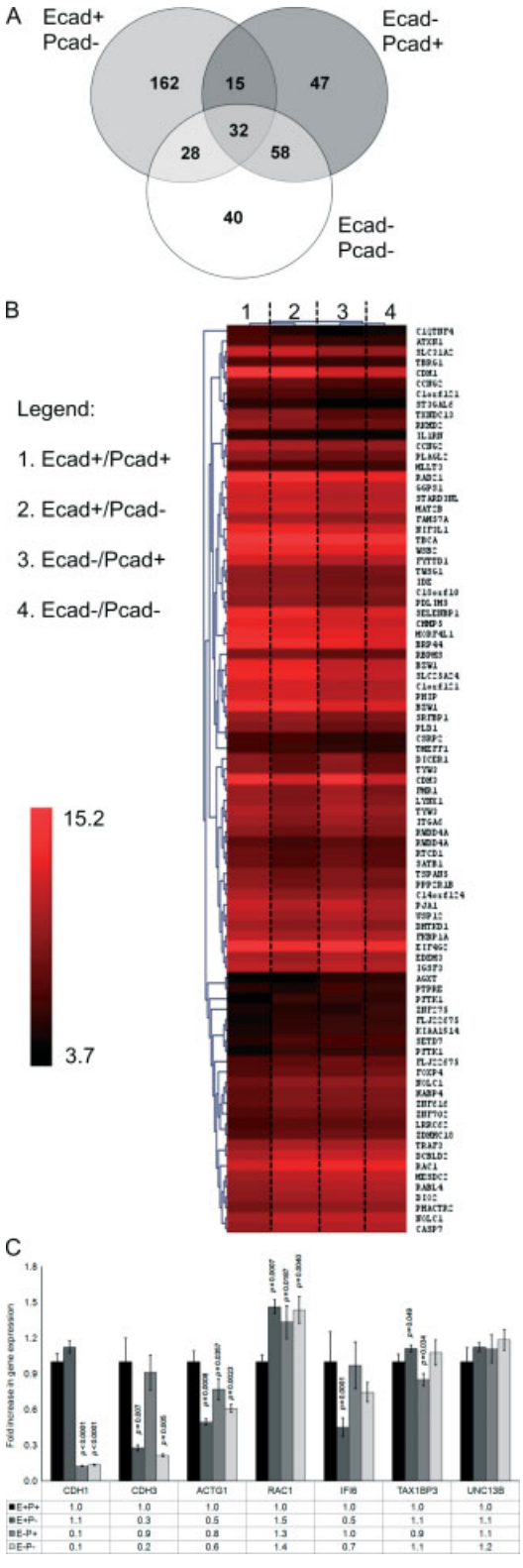

Figure 4. Gene transcriptional modulation for E- and P-cadherin co-expression in BT20 breast cancer cells. (A) Venn diagram depicting the genes with modified expression levels in the different cell lines compared with the parental cell line. (B) Unsupervised hierarchical clustering of the gene expression patterns induced by E- or P-cadherin expression. Columns represent microarray experiments and rows represent the expression of genes modified at least 2-fold with respect to control cells. (C) Validation of the microarray data of selected genes by qRT-PCR. Bars represent mean gene expression ± SEM (mRNA levels relative to control GAPDH transcript) from three independent experiments.

injected 10^6 BT20 cells in the mammary fat pad of nude mice. Visible and palpable nodules were already observed 14 days after cell inoculation. After 35 days, tumours showed a characteristic pattern of basal-like breast carcinomas, being solid, with pushing margins, high histological grade, and, as expected, expressing membrane E- and P-cadherin (Figure 5A). Subsequently, we inoculated control ($Ecad^+/Pcad^+$), $Ecad^+/Pcad^-$, $Ecad^-/Pcad^+$ or $Ecad^-/Pcad^-$ cells and monitored tumour formation and growth for 35 days. $Ecad^+/Pcad^+$ tumours showed a significant increase in the tumour volume, compared with $Ecad^+/Pcad^-$ and $Ecad^-/Pcad^+$ tumours (Figures 5B and 5C) at 28 and 35 days (Figures 5D and 5E). Curiously, $Ecad^-/Pcad^-$ tumour cells showed similar tumour growth to $Ecad^+/Pcad^+$ breast cancer cells.

Prognostic significance of E- and P-cadherin co-expression in primary breast carcinomas

Since $Ecad^+/Pcad^+$ cells show increased invasive and tumourigenic behaviour, but also considering that distinct cadherins can eventually interfere with each other at the cell membrane [36,37], we decided to determine whether both cadherins co-localize in normal and tumour cells.

In normal breast, E-cadherin is expressed in luminal and myoepithelial cells, whereas P-cadherin expression is restricted to the myoepithelium (Figure 6A). In the case of breast carcinomas, the series included 24.4% of the cases with membrane P-cadherin (114/452) and 94.2% with membrane E-cadherin expression (426/452, Supplementary Table 2). Considering the combined expression of epithelial cadherins, 23.5% of the tumours were $Ecad^+/Pcad^+$, 0.9% were $Ecad^-/Pcad^+$, 70.7% were $Ecad^+/Pcad^-$, and 4.9% were $Ecad^-/Pcad^-$ (Supplementary Table 2). Although in some cases the intensity of E-cadherin membrane expression was reduced in cancer cells with P-cadherin expression (as in normal myoepithelial cells), in the majority of the cases E-cadherin expression was maintained. Indeed, both cadherins co-localized at the cell membrane and were positive for *in situ* PLA signals; as expected, $Ecad^+/Pcad^-$ tumours were negative for *in situ* PLA signals (Figure 6A).

$Ecad^+/Pcad^+$ tumours showed a statistically significant association with high histological grade ($p = 0.031$); however, no significant association was found with tumour size and nodal status (Table 2). $Ecad^+/Pcad^+$ tumours were also significantly associated with negativity for hormonal receptor expression [ERα and PR, respectively ($p < 0.001$ and $p = 0.001$)] and positively associated with HER2 ($p = 0.017$) and basal-like marker expression [CK14 ($p < 0.001$), CK5 ($p < 0.001$), vimentin ($p < 0.001$), and EGFR ($p < 0.001$)] (Table 2).

Disease-free (DFS) and overall survival (OS) also differed significantly between tumours with distinct patterns of E- and P-cadherin expression, which were significantly lower for patients with $Ecad^+/Pcad^+$

Table 1. List of the genes significantly altered after E- and P-cadherin silencing in BT20 breast cancer cells*

Gene ontology	E+/P+	E+/P−	E−/P+	E−/P−	p value
CDH1	1.0	0.9	0.1	0.1	3.2E-06
CDH3	1.0	0.2	1.1	0.2	9.0E-07
Small GTPase-mediated signal transduction					
RABL4	1.0	2.3	2.6	2.3	4.4E-05
C1ORF89	1.0	2.0	1.3	1.6	1.6E-02
GRLF1	1.0	2.1	1.5	1.8	5.8E-03
RGS19	1.0	2.8	–	–	1.1E-04
RHOB	1.0	1.8	–	–	6.3E-03
MYO9B	1.0	2.0	–	–	6.4E-03
TAX1BP3	1.0	2.1	1.1	1.6	5.2E-03
RAPGEF1	1.0	2.1	–	–	8.5E-03
ARL4C	1.0	1.9	–	–	4.6E-03
ARL5B	1.0	0.5	0.5	0.5	7.0E-03
Regulation of cell migration					
PLD1	1.0	1.1	0.5	0.4	2.3E-06
KISS1R	1.0	–	0.4	–	9.9E-03
NISCH	1.0	2.2	2.1	2.0	4.1E-04
GAB1	1.0	0.8	0.5	0.4	1.2E-03
RAC1	1.0	1.9	2.2	1.8	3.1E-02
ARHGAP8	1.0	0.7	0.5	0.5	2.8E-02
Actin cytoskeleton organization					
ACTG1	1.0	0.4	–	–	1.3E-03
TRIOBP	1.0	0.5	0.7	0.6	1.1E-02
CDC42BPG	1.0	1.9	–	–	4.0E-03
GNA11	1.0	0.5	0.6	0.5	7.0E-03
BIN3	1.0	0.6	0.6	0.5	7.0E-03
Apoptosis					
AKT1	1.0	2.2	1.4	2.0	4.3E-02
CASP7	1.0	2.2	2.3	2.1	4.1E-04
CASP9	1.0	0.5	0.8	0.7	1.9E-03
IFI6	1.0	0.5	0.9	1.3	2.5E-02
FADD	1.0	2.2	1.0	1.5	2.4E-02
ARHGEF9	1.0	2.3	1.5	2	2.2E-03
MX1	1.0	0.4	0.7	0.7	1.9E-02
UNC13B	1.0	2.0	1.4	1.5	3.4E-03
TRAF3	1.0	2.0	1.7	1.9	1.6E-04
MAPK8	1.0	0.9	0.5	0.4	5.6E-03
MAL	1.0	1.1	2.5	2.2	2.3E-02
GCH1	1.0	0.8	0.6	0.5	6.5E-03
PLAGL2	1.0	0.6	0.4	0.4	2.7E-04

*mRNA profiles were determined for the BT20 breast cancer cell model after E- and P-cadherin KD. The profiles identified 382 genes, whose expression was deregulated (DEGs) ($p < 0.01$; > 2-fold increase/decrease), in at least one of the four groups in relation to the control cell line ($Ecad^+/Pcad^+$). The genes represented are the ones that were differentially expressed in functional groups and in enriched signalling pathways, among the distinct BT20 cell lines. These genes are organized according to gene ontology assignment, and the values represent the fold increase/decrease in gene expression when compared with control cells ($Ecad^+/Pcad^+$).

or $Ecad^-/Pcad^-$ tumours [$p = 0.043$ (Figure 6B) and $p = 0.027$ (Figure 6C), respectively]. Univariate analysis showed that E- and P-cadherin co-expression was a significant prognostic factor for DFS and OS, since these cases carried an increased risk of recurrence (HR = 1.44, 95% CI = 1.04–1.99, $p = 0.029$) or death (HR = 1.53, 95% CI = 1.09–2.15, $p = 0.014$), compared with tumours expressing only E-cadherin (Table 3). The same analysis performed for negative tumours for both cadherins did not show a significant association with patient prognosis. Indeed, single E-cadherin expression was not found to be a significant prognostic marker for DFS or OS ($p = 0.216$ and $p = 0.280$, respectively), whereas P-cadherin expression was a significant prognostic predictor for OS (HR = 1.45, 95% CI = 1.04–2.02, $p = 0.028$), but not for DFS ($p = 0.059$, Table 3). Additionally, the multivariate analysis, with models including tumour size, grade, and nodal status, demonstrated the independent value of E- and P-cadherin co-expression as a prognostic factor of patient outcome in breast cancer, being significantly associated with worse breast cancer OS (HR = 1.51, 95% CI = 1.03–2.22, $p = 0.036$, Table 3).

Discussion

E-cadherin and P-cadherin mediate epithelial cell–cell adhesion and harbour opposite roles in cancer. Decreased E-cadherin expression is related to invasiveness [4,5,38–40], and the mechanisms underlying its loss-of-function have already been studied [8,38]. However, in some cancer models, E-cadherin expression is maintained, being associated with increased

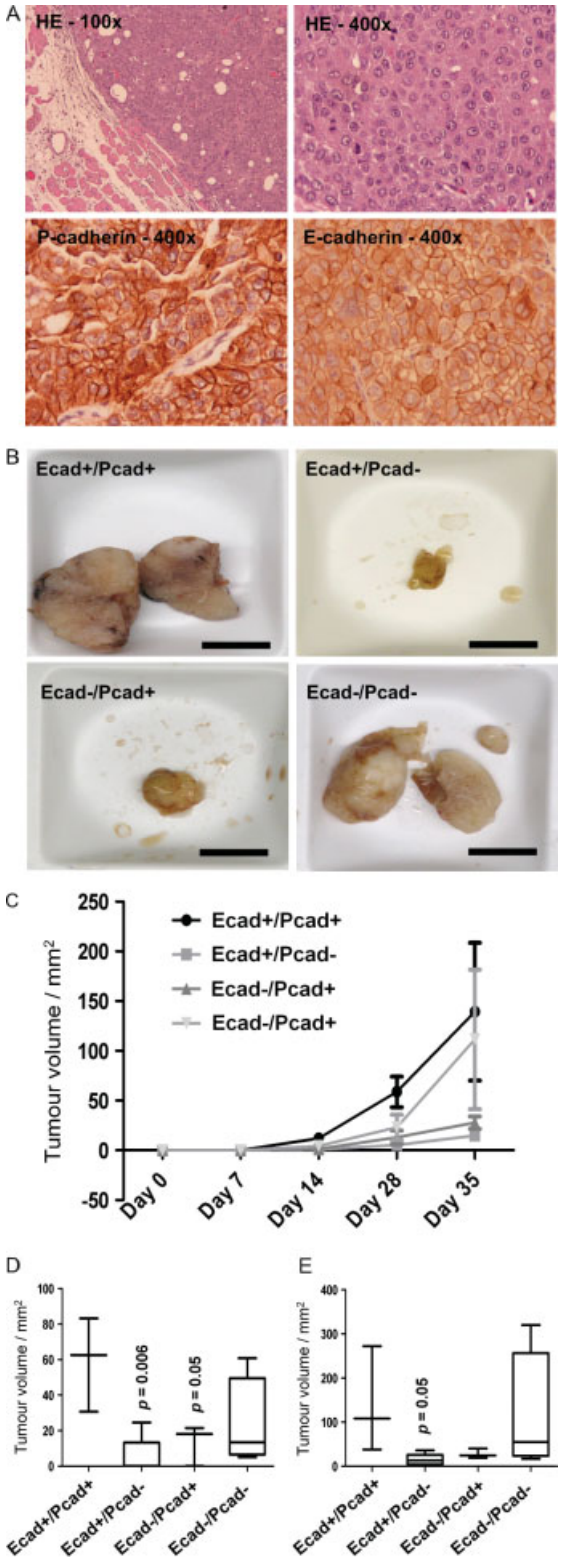

Figure 5. Effect of E and P-cadherin co-expression on *in vivo* tumour growth. Nude mice were injected in the mammary fat pad with the different BT20 cell lines generated. (A) H&E staining and immunohistochemistry for P- and E-cadherin of an Ecad+/Pcad + xenografted tumour. (B) Representative images of the tumours formed 35 days after tumour cell inoculation. Scale bar = 1cm. (C) Mean tumour volume in nude mice ($n = 4$ mice per group). (D, E) Box plot for tumour volume 24 days (D) or 35 days (E) after tumour cell inoculation. Error bars represent upper 95% confidence intervals. p values were calculated using two-sided Student t-tests; statistically significant p values are indicated.

cell survival and metastatic spread [41–45]. It was shown that E-cadherin positive status is not always predictive of good prognosis in breast cancer [46].

Moreover, local advanced inflammatory breast cancer [20,47] and some highly metastatic breast cancer cells, such as the 4T1 cell model [48,49], maintain normal membrane E-cadherin expression. Interestingly, these tumours/cell models show the aberrant expression of P-cadherin (Supplementary Table 1). Actually, during breast cancer progression, P-cadherin is overexpressed and is associated with worse patient survival [14,15,20,50–52]; however, the cellular mechanism by which P-cadherin has an important role in cancer remained elusive. We hypothesized that E- and P-cadherin co-expression could be involved in a more aggressive biological behaviour of breast cancer cells, due to interaction of both molecules at the cellular membrane, interfering with the establishment of a strong adhesion complex. Cadherins normally form tight complexes with catenins, which functionally link them to the actin cytoskeleton. Changes in the structure of the adhesion complex and alterations in the interaction between molecules of the complex with the actin cytoskeleton have been suggested to play a role in cell–cell adhesion deregulation, as well as a mechanism to trigger specific signalling pathways that induce cancer cell invasion [22,25,42,53]. In fact, p120ctn is essential for the stability of cadherins [11,54] and its subcellular localization plays an important role in motility-promoting signals [55–57], since the JMD (which is the domain where it binds) was found to be important to P-cadherin pro-invasive activity [58–62]. We therefore analysed the interaction between E- and P-cadherin with catenins, demonstrating that Ecad$^+$/Pcad$^+$ cells showed a deregulated cadherin/catenin complex at the cellular membrane. In contrast, cells expressing only one of the cadherins showed an increase in the cadherin/catenin membrane interactions. Our results indicate that E- and P-cadherin heterodimers are not efficient in the stabilization of a strong cadherin/catenin complex at the cellular membrane and, therefore, cells show an aberrant cell behaviour.

This hypothesis was confirmed using functional *in vitro* assays and microarrays. In fact, Ecad$^+$/Pcad$^+$ cells had a higher invasive capacity than cells expressing only one of the cadherins. Indeed, P-cadherin functions as an invasion suppressor when it is the only cadherin expressed at the cell membrane [24,25]; however, when co-expressed with E-cadherin, it has a pro-invasive function, which explains the poor prognosis of patients with tumours co-expressing both molecules [19,21,23]. Microarrays confirmed that the gene signature was also different, depending on which cadherin was expressed, explaining in part the results observed in *in vitro* assays. Sarrió *et al* have previously tried to tackle this issue; they demonstrated that E- and P-cadherin independent expression has similar functional consequences in the suppression of the invasive behaviour of breast

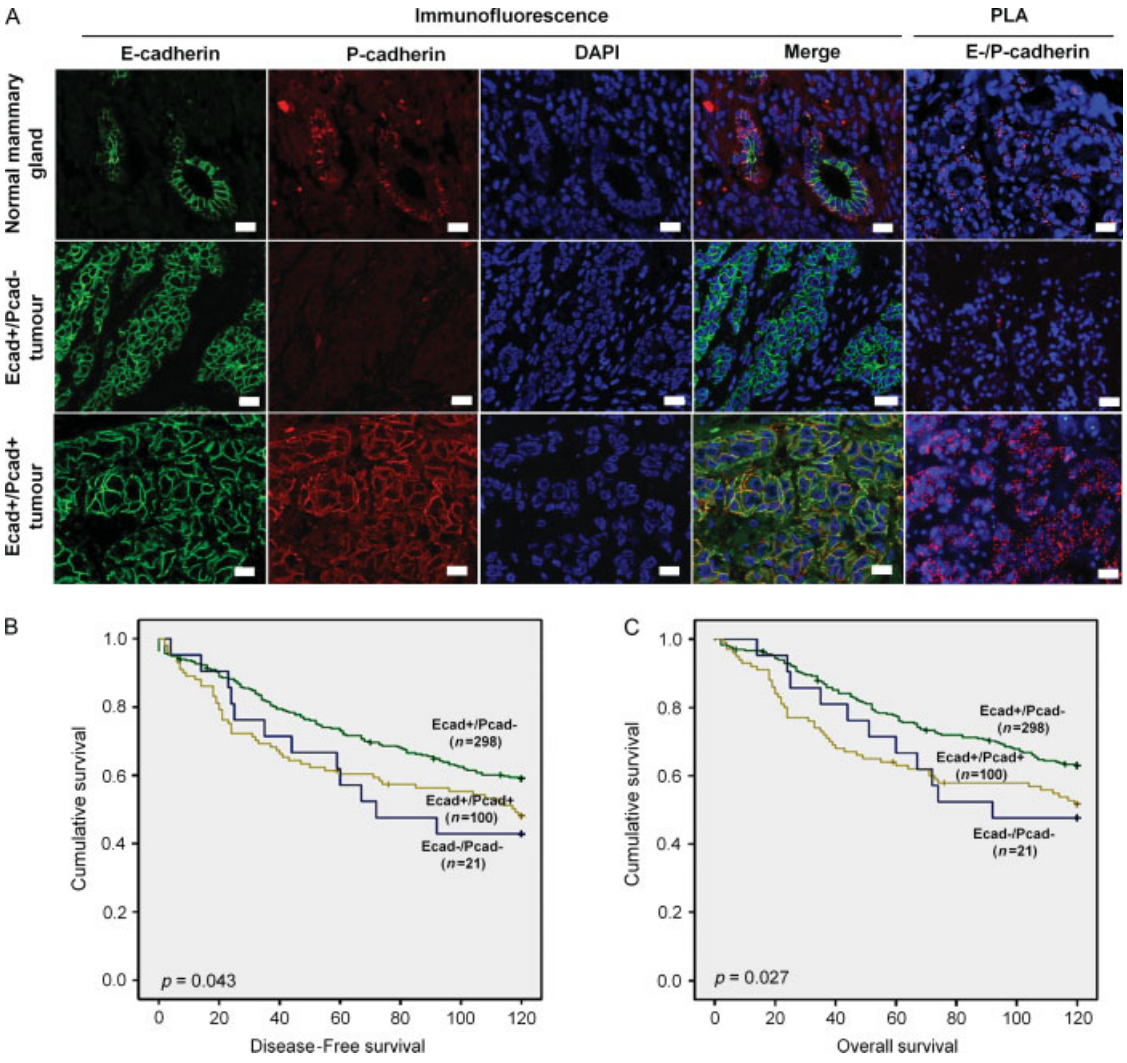

Figure 6. P-cadherin and E-cadherin expression in primary breast tumours. (A) Immunofluorescence and *in situ* proximity ligation assay for E-cadherin and P-cadherin expression were analysed in normal breast and tumour samples. (B, C) Kaplan–Meier curves showing the probability of disease-free survival (B) and overall survival (C) of patients harbouring invasive breast carcinomas with distinct E- and P-cadherin expression patterns. Censored data correspond to patients that left the study before the end of the considered follow-up. *p* values less than 0.05 were considered statistically significant. Since only four tumours showed positivity for P-cadherin without E-cadherin expression, and to avoid a bias in the statistical method used, this group of patients was not considered in the survival analysis.

cancer cells, as well as being able to induce both common gene expression programmes [25]. However, we focused on E- and P-cadherin co-expression and found that the simultaneous expression of the cadherins promotes aggressive biological behaviour and different gene expression profiles, compared with the expression of each cadherin alone. We still proved that $Ecad^+/Pcad^+$ cells have increased *in vivo* tumour growth.

Interestingly, we observed a dominant effect of P-cadherin in triggering cell migration. P-cadherin's role in cell migration has been previously described [19,22] and gene expression profiles suggest that these specific effects could be explained by alterations in small GTPase-mediated signal transduction. Indeed, increased Rac activity was observed in $Ecad^+/Pcad^+$ cells. Although the microarray results show that *Rac1* mRNA expression increases after siRNA silencing, this can mean that there is a gene transcription response, but no activation of this protein by GEFs. Interestingly, the microarrays also showed that the *TRIOBP* gene, which encodes a protein that interacts with TRIO (a Rac-GEF protein), is expressed in higher amounts in control cells than in cells transfected with one or both siRNAs. Based on these preliminary results, this pathway will be further explored in the future as a possible molecular mechanism activated in $Ecad^+/Pcad^+$ cells.

We have shown that P-cadherin is a regulator of cell survival, since a significant increase in the cell death of P-cadherin silenced cells was observed after an apoptotic stimulus. This effect was partially counteracted by adding a pan-caspase inhibitor, suggesting that caspase activation has a crucial, but not unique, role in this P-cadherin-induced mechanism of cell death. This effect of P-cadherin as a potential survival factor was strongly supported by microarray data, which showed that genes involved in intrinsic and extrinsic apoptotic signalling are enriched in $Ecad^+/Pcad^-$ cells.

Table 2. Correlation between E- and P-cadherin co-expression with clinico-pathological parameters and molecular markers*

			E- and P-cadherin expression				
		N	Ecad$^-$/Pcad$^-$ (%)	Ecad$^+$/Pcad$^-$ (%)	Ecad$^-$/Pcad$^+$ (%)	Ecad$^+$/Pcad$^+$ (%)	*p* value
Tumour size	T1: < 2 cm	100	1 (5.0)	80 (28.8)	1 (25.0)	18 (18.8)	0.15
	T2: 2–5 cm	237	14 (70.0)	160 (57.5)	2 (50.0)	61 (63.5)	
	T3: > 5 cm	61	5 (25.0)	38 (13.7)	1 (25.0)	17 (17.7)	
	Missing	69					
Nodal status	Negative	154	4 (28.6)	114 (45.6)	2 (66.7)	34 (38.6)	0.359
	Positive	201	10 (71.4)	136 (54.4)	1 (33.3)	54 (61.4)	
	Missing	112					
Histological grade	1	78	4 (19.0)	62 (20.5)	2 (50.0)	10 (9.7)	*0.031*
	2	130	8 (38.1)	95 (31.5)	1 (25.0)	26 (25.2)	
	3	222	9 (42.9)	145 (48.0)	1 (25.0)	67 (65.1)	
	Missing	37					
ERα	Negative	151	7 (31.8)	78 (24.5)	2 (50.0)	64 (61.0)	*< 0.001*
	Positive	298	15 (68.2)	240 (75.5)	2 (50.0)	41 (39.0)	
	Missing	18					
PR	Negative	230	13 (61.9)	144 (45.1)	2 (50.0)	71 (67.0)	*0.001*
	Positive	220	8 (38.1)	175 (54.9)	2 (50.0)	35 (33.0)	
	Missing	17					
HER2	Negative	381	21 (95.5)	276 (87.6)	3 (75.0)	81 (76.4)	*0.017*
	Positive	66	1 (4.5)	39 (12.4)	1 (25.0)	25 (23.6)	
	Missing	20					
EGFR	Negative	429	21 (95.5)	316 (99.1)	3 (75.0)	89 (84.0)	*< 0.001*
	Positive	22	1 (4.5)	3 (0.9)	1 (25.0)	17 (16.0)	
	Missing	16					
CK5	Negative	385	21 (95.5)	292 (91.5)	2 (50.0)	70 (66.0)	*< 0.001*
	Positive	66	1 (4.5)	27 (8.5)	2 (50.0)	36 (34.0)	
	Missing	16					
CK14	Negative	427	21 (95.5)	312 (97.8)	3 (75.0)	91 (85.8)	*< 0.001*
	Positive	24	1 (4.5)	7 (2.2)	1 (25.0)	15 (14.2)	
	Missing	16					
Vimentin	Negative	370	15 (71.4)	281 (89.2)	3 (75.0)	71 (67.0)	*< 0.001*
	Positive	76	6 (28.6)	34 (10.8)	1 (25.0)	35 (33.0)	
	Missing	21					

*Contingency tables and the chi-square test were used to estimate the relationship between E- and P-cadherin staining patterns and the several parameters analysed. The ANOVA test was used to evaluate the differences in tumour size, considering a confidence interval of 95%. Statistical analyses were performed by SPSS 15.0 software. *p* values less than 0.05 were considered statistically significant (indicated in italics in the table).

Table 3. Univariate and multivariate Cox proportional hazard analysis for classical prognostic factors, such as tumour size, lymph node status, and tumour grade, as well as for E-cadherin, P-cadherin, and their co-expression*

		Univariate Cox proportional hazard analysis				Multivariate Cox proportional hazard analysis			
		Disease-free survival		Overall survival		Disease-free survival		Overall survival	
Variable	Evaluation	HR (95% CI)	*p* value	HR (95% CI)	*p* value	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Tumour size	T1 (≤ 2 mm)	1		1		1		1	
	T2 (2 < T ≤ 5 mm)	2.49 (1.59–3.91)	< 0.001	2.73 (1.67–4.50)	< 0.001	1.61 (0.96–2.71)	0.068	1.87 (1.06–3.32)	0.031
	T3 (> 5 mm)	4.62 (2.76–7.73)	< 0.001	5.25 (3.02–9.15)	< 0.001	2.95 (1.65–5.29)	< 0.001	3.54 (1.90–6.71)	< 0.001
Lymph node status	Negative	1		1		1		1	
	Positive	2.41 (1.70–3.41)	< 0.001	2.64 (1.81–3.85)	< 0.001	1.89 (1.30–2.77)	0.001	2.04 (1.35–3.08)	0.001
Tumour grade	Grade I	1		1		1		1	
	Grade II	1.36 (0.84–2.19)	0.21	1.49 (0.90–2.46)	0.117	1.26 (0.64–2.45)	0.501	1.25 (0.62–2.50)	0.535
	Grade III	1.91 (1.24–2.95)	0.003	1.94 (1.22–3.08)	0.005	1.9 (1.02–3.54)	0.043	1.66 (0.87–3.12)	0.122
E-cadherin expression	Positive	1		1		1		1	
	Negative	1.45 (0.81–2.60)	0.216	1.41 (0.77–2.60)	0.269	1.066 (0.46–2.46)	0.88	0.8 (0.32–1.99)	0.627
P-cadherin expression	Negative	1		1		1		1	
	Positive	1.36 (0.99–1.87)	0.059	1.45 (1.04–2.02)	0.028	1.35 (0.94–1.95)	0.109	1.51 (1.03–2.22)	0.036
E-/P-cadherin expression	Positive/negative	1		1		1		1	
	Negative/negative	1.59 (0.88–2.88)	0.124	1.58 (0.85–2.93)	0.149	1.06 (0.46–2.46)	0.88	0.8 (0.32–1.99)	0.627
	Positive/positive	1.44 (1.04–1.99)	0.029	1.53 (1.09–2.15)	0.014	1.35 (0.94–1.95)	0.109	1.51 (1.03–2.22)	0.036

*This analysis allows the risk (hazard ratios and the corresponding 95% confidence interval, CI) to be predicted for disease-free and overall survival of breast cancer patients. A significance level of 5% was considered.

We have demonstrated that P-cadherin is a putative cancer stem cell marker in breast cancer [63], since its expression, in E-cadherin wild-type cells, promoted an increase in anoikis-resistant cells with anchorage-independent growth. Curiously, induction of anoikis resistance has also been observed after loss of E-cadherin function [64] or by cytoplasmic localization of p120ctn [12]. Ecad$^+$/Pcad$^+$ tumours

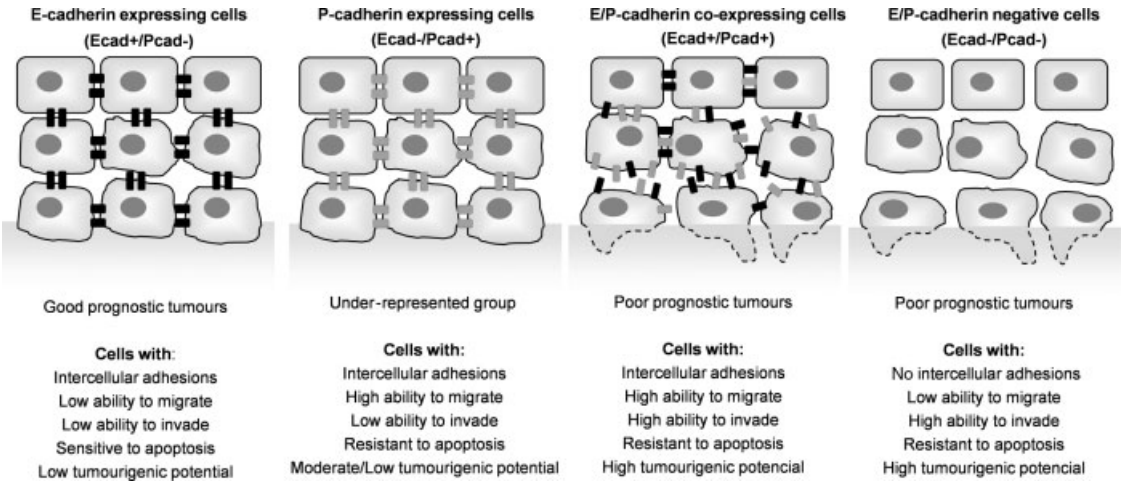

Figure 7. Schematic representation of the different types of breast cancer in what concerns cadherin expression. Tumours that maintain normal E-cadherin expression present a good prognosis. These cancer cells normally show intercellular adhesions, low ability to migrate, low ability to invade, and are still sensitive to apoptosis. The tumours that only express P-cadherin are uncommon and the breast cancer cells show intercellular adhesions, are resistant to apoptosis, and although they present high ability to migrate, they are not able to invade the extracellular matrix. On the other hand, the tumours negative for both cadherins show a poor prognosis and the cancer cells do not show intercellular adhesions, are resistant to apoptosis, and present a high ability to invade; however, these tumour cells are less motile. Finally, in the breast cancer cells with co-expression of cadherins, intercellular adhesion is maintained, although the tumour cells present an increase in the ability to migrate, to invade, and show resistance to apoptosis. Also, tumours co-expressing E- and P-cadherin present a poorer prognosis compared with tumours that show exclusive E-cadherin expression.

show significantly decreased patient DFS and OS. The evaluation of P-cadherin expression, in an E-cadherin$^+$ background, has important value in the prognostic risk assessment of patient recurrence and death [65–68]. Additionally, multivariate analysis showed that co-expression of cadherins is an independent prognostic marker for OS. With this study, P-cadherin can be considered a robust independent biomarker of poor prognosis in breast cancer.

In conclusion, tumour cells acquire features that give them an advantage to survive in a hostile environment, migrate, and invade [69]. Based on our data, we conclude that Ecad$^+$/Pcad$^+$ cells versus cells that express only one of the cadherins show a more aggressive biological behaviour (Figure 7). Our results also show, for the first time, that P-cadherin overexpression in an E-cadherin wild-type context is an alternative mechanism for cancer invasion, disrupting the interaction between E-cadherin and intracellular catenins and leading to alterations in biological behaviour and the gene expression profile of breast cancer cells. This work reinforces the importance of P-cadherin expression as a prognostic factor for breast cancer patients and supports the development of new therapeutics to control aggressive carcinomas co-expressing both cadherins.

Acknowledgments

This work was supported by a scientific project (Ref: PTDC/SAU-GMG/120049/2010), three research grants (ASR: SFRH/BPD/75705/2011; AA: SFRH/BPD/73247/2010; BS: SFRH/BD/69353/2010), and Programa Ciência 2007 (JP) and Ciência 2008 (LC) (Contratação de Doutorados para o SCTN – financiamento pelo POPH – QREN – Tipologia 4.2 – Promoção do Emprego Cientrfico, comparticipado pelo Fundo Social Europeu e por fundos nacionais do MCTES), all funded by Fundação para a Ciência e Tecnologia (Portugal). IPATIMUP is an Associate Laboratory of the Portuguese Ministry of Science, Technology and Higher Education and is partially supported by FCT.

Author contribution statement

ASR and JP conceived experiments, analysed the data, and were involved in writing the paper. ASR, BS, LC, NM, SR, AA, ARN, and RG carried out experiments. JFC was involved in data collection. OS, MAS, RS, and FS were involved in data analysis and interpretation. All authors had final approval of the submitted and published versions.

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article.

Supplementary materials and methods.

Table S1. Summary of the results obtained in different studies that assessed the invasion role of P-cadherin in several cell lines, taking into account the pattern of expression of classical cadherins (namely E- and N-cadherin).

Table S2. Summary of the clinical, pathological, and immunohistochemical features of the studied series of 467 invasive breast carcinomas, including E- and P-cadherin expression.

Figure S1. Functional evaluation of E- and P-cadherin expression in SUM149 breast cancer cell line.

Figure S2. Quantification of specific protein–protein interactions in breast cancer cells after E- and P-cadherin transcription silencing by specific siRNAs in SUM149.

Figure S3. BrdU proliferation assay.

Figure S4. Validation of microarray data of selected genes by qRT-PCR in SUM149 breast cancer cells.

Figure S5. Activity of Rac and Rho in BT20 breast cancer cells.

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CCAAT/Enhancer Binding Protein β (C/EBPβ) Isoforms as Transcriptional Regulators of the Pro-Invasive *CDH3*/P-Cadherin Gene in Human Breast Cancer Cells

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Abstract

P-cadherin is a cell-cell adhesion molecule codified by the *CDH3* gene, which expression is highly associated with undifferentiated cells in normal adult epithelial tissues, as well as with poorly differentiated carcinomas. In breast cancer, P-cadherin is frequently overexpressed in high-grade tumours and is a well-established indicator of aggressive tumour behaviour and poor patient prognosis. However, till now, the mechanisms controlling *CDH3* gene activation have been poorly explored. Since we recently described the existence of several CCAAT/Enhancer Binding Protein β (C/EBPβ) transcription factor binding sites at the *CDH3* promoter, the aim of this study was to assess if the distinct C/EBPβ isoforms were directly involved in the transcriptional activation of the *CDH3* gene in breast cancer cells. DNA-protein interactions, mutation analysis and luciferase reporter assay studies have been performed. We demonstrated that C/EBPβ is co-expressed with P-cadherin in breast cancer cells and all the three isoforms function as transcriptional regulators of the *CDH3* gene, directly interacting with specific regions of its promoter. Interestingly, this transcriptional activation was only reflected at the P-cadherin protein level concerning the LIP isoform. Taken together, our data show that *CDH3* is a newly defined transcriptional target gene of C/EBPβ isoforms in breast cancer, and we also identified the binding sites that are relevant for this activation.

Citation: Albergaria A, Resende C, Nobre AR, Ribeiro AS, Sousa B, et al. (2013) CCAAT/Enhancer Binding Protein β (C/EBPβ) Isoforms as Transcriptional Regulators of the Pro-Invasive *CDH3*/P-Cadherin Gene in Human Breast Cancer Cells. PLoS ONE 8(2): e55749. doi:10.1371/journal.pone.0055749

Editor: Olivier de Wever, Ghent University, Belgium

Received July 13, 2012; **Accepted** December 31, 2012; **Published** February 6, 2013

Funding: This work was supported by research grants (SFRH/BPD/73247/2010 to A.A., SFRH/BD/46504/2008 to C.R., SFRH/BPD/75705/2011 to A.S.R. and SFRH/BD/69353/2010 to B.S.), from the Portuguese Foundation for Science and Technology (FCT). J.P. is supported by Programa Ciência 2007 (POPH - QREN - Tipology 4.2). Institute of Molecular Pathology and Immunology of Porto University (IPATIMUP) is an Associate Laboratory of the Portuguese Ministry of Science, Technology and Higher Education and is partially supported by FCT. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The molecular changes that occur during breast cancer progression, which include the amplification/overexpression of transcription factors, can disrupt the delicate balance between cell proliferation, differentiation and apoptosis. C/EBPβ is one of those transcription factors, which has been implicated in cell cycle regulation, playing an important role in mammary gland development and oncogene-induced breast tumorigenesis [1–4]. Encoded by an intronless gene, C/EBPβ is expressed as distinct protein isoforms, which can accomplish distinct biological and regulatory functions, ultimately leading to gene transactivation [5]. The longer C/EBPβ proteins (liver-enriched transcriptional activating proteins, LAP1 and LAP2) regulate proliferation and differentiation of many cell types [6]; the shorter protein product (liver-enriched transcriptional inhibitory protein, LIP) lacks the transactivation domain and acts mainly as a dominant-negative [7]. AS LAP isoforms, LIP also binds to the consensus sequences within genomic DNA, sometimes even with a higher affinity than the other C/EBPβ isoforms [6,7]. In fact, LIP inhibits the transcriptional activity of LAPs by competing for the same consensus binding sites or by forming inactive heterodimers with them. However, some emerging evidence suggest that LIP can also act as a transcriptional activator in some cellular contexts [5].

In breast, C/EBPβ most likely contributes to tumorigenesis through significant elevations in the LIP:LAP ratio, mostly observed in ER-negative, highly proliferative and metastatic mammary tumours, usually associated with a poor patient prognosis [8]. Indeed, LIP isoform overexpression has been associated to a lack of contact inhibition, resulting in proliferation and *foci* formation in epithelial breast cancer cell lines [9]. It has been hypothesized that aberrant expression of C/EBPβ-LIP isoform may contribute to an increased growth rate and result in a more proliferative and aggressive breast carcinoma.

P-cadherin, a classical cadherin encoded by the *CDH3* gene [10], has been explored by our group for several years and has been also extensively associated with breast tumour aggressiveness. This protein was found to be aberrantly expressed in 20–40% of invasive ductal carcinomas, being strongly associated with proliferative lesions of high histological grade, decreased cell

polarity and poor patient survival [11–16]. At the *in vitro* level, we demonstrated that P-cadherin overexpression induces invasion [14], motility and migration of wild-type E-cadherin expressing breast cancer cells, through the secretion of pro-invasive factors, such as matrix metalloproteinase (MMP)-1 and MMP-2 [17]. In fact, P-cadherin-associated functions in breast cancer have been widely studied, which reflects the growing importance of this cadherin in human breast cancer biology and prognosis.

However, the mechanisms controlling its overexpression in breast cancer have only recently started to be unrevealed [11,18]. In non-cancer models, *CDH3* promoter was shown to be genetically regulated through direct binding of transcription factors, such as p63 [19] and β-catenin [20]. Gorski and collaborators also demonstrated that BRCA1 and c-Myc form a repressor complex on *CDH3* promoter and on other promoters of specific basal genes, representing a potential mechanism to explain the overexpression of key basal markers in BRCA1-deficient breast tumours [21]. Additionally, we established a direct link between P-cadherin overexpression and the lack of oestrogen receptor (ER)-signalling in breast cancer cells, categorizing *CDH3* as a putative ER-repressed gene [14]. In 2010, we described a regulatory mechanism whereby a selective ER-downregulator is able to up-regulate P-cadherin expression in MCF-7/AZ breast cancer cells through chromatin remodelling at *CDH3* promoter level [18]. This epigenetic process was accomplished by the induction of high levels of the active chromatin mark H3K4me2 and a consequent de-repression of the *CDH3* promoter, which exposed a high number of putative C/EBPβ transcription binding sites [18]. The induction of *CDH3* promoter activity by C/EBPβ was also confirmed by reporter assays, as well as its expression association with worse prognosis of breast cancer patients [18].

However, since the mechanistic link and the consequent transcriptional regulatory relevance of C/EBPβ proteins on *CDH3* gene were not demonstrated, in the present study we revealed that C/EBPβ isoforms are indeed transcriptional regulators of P-cadherin, directly interacting with conserved and specific regions of the *CDH3* promoter. Interestingly, we show that this transcriptional activation is reflected in the P-cadherin protein levels, especially for the LIP isoform. We conclude that *CDH3* is a newly defined transcriptional target gene of C/EBPβ in breast cancer.

Materials and Methods

Antibodies

The following primary anti-human antibodies were used for Western Blot and/or Immunohistochemistry against: P-cadherin (BD Transduction Biosciences, Lexington, KY), C/EBPβ (Santa Cruz Biotechnology, CA), β-actin (Santa Cruz Biotechnology) and β-tubulin (Sigma-Aldrich, St. Louis, NO). Technical conditions are described in Table S1 (Supporting Information). Anti-mouse and anti-goat horseradish peroxidase-conjugated secondary antibodies were used for WB [HRP-conjugated, dilutions: 1:2000] (Santa Cruz Biotechnology). For chromatin immnunoprecipitation (ChIP) assays, the following antibodies were used: anti-C/EBPβ (C-19, Santa Cruz Biotechnology), and two control IgGs (Active Motif, CA and Santa Cruz Biotechnology).

Promoter Vectors and cDNA Constructs

The pLENTI-C/EBPβ expression vectors (C/EBPβ-LAP1, C/EBPβ-LAP2 and C/EBPβ-LIP) were generated according to the human *CEBPB* nucleotide sequence obtained from Ensembl and Pubmed databases. Oligonucleotide primer sequences for LAP1, LAP2, and LIP isoforms are listed in Table S2 (see Supporting Information).

CEBPB cDNA was obtained from total RNA extracted from the gastric cancer cell line AGS, and amplified for each *CEBPB* isoform using HotStart Taq DNA Polymerase (Qiagen, Cambridge, MA). Amplification was performed for 35 cycles as follows: denaturation at 95°C for 1 minute, annealing at 60°C for LAP1 and LAP2 and 58°C for LIP for 1 minute, and extension at 68°C for 2 minutes per cycle. PCR products for each isoform were separated by electrophoresis in a 1.5% agarose gel and bands were sequenced using the ABI Prism Dye Terminator Cycle Sequencing Kit (Perkin-Elmer, Beaconsfield, UK). To validate the isoforms nucleotide sequence, amplified products were purified through Sepharose (GE Healthcare, Waukesha, WI) and sequenced on both strands on an ABI Prism 3100 automated sequencer (Perkin-Elmer). PCR products were inserted into the mammalian expression vector pLENTI6/V5 Directional (Invitrogen, Ltd, Paisley, UK), using manufacturer instructions, and incorporated into chemically competent TOP10 *E. coli* (Invitrogen). Transformed bacteria were grown overnight in ampicillin-supplemented LB-Agar (Applichem, Germany). Plasmid DNA from transformed *E. coli* cells was sequenced to check the orientation and nucleotide sequence for each *CEBPB* isoform.

The human full-length *CDH3*-luciferase vector was generated by our group, as previously described [18]. Normalization pRL-CMV Renilla Luciferase Control Reporter Vector was purchased to Promega (Promega Corporation, Madison, WI).

Immunohistochemistry

Double immunostaining for C/EBPβ and P-cadherin was performed in 3 μm sections of 23 formalin-fixed paraffin-embedded (FFPE) invasive breast carcinomas that have previously showed strong expression of both proteins, in order to illustrate their consistent cellular co-localization. Standard immunohistochemistry was performed as previously described [16]. For the reaction, we used the Envision G2 Double-stain (DakoCytomation, Glostrup, Denmark), according to manufacturer instructions. Specific conditions used for C/EBPβ and P-cadherin are listed in Table S1. FFPE sections from normal breast gland, skin or normal gastric mucosa were used as positive controls for C/EBPβ and P-cadherin. Negative controls were performed by replacing the primary antibody with PBS/non-immune serum.

The present study was conducted under the national regulative law for the usage of biological specimens from tumour banks, where the samples are exclusively available for research purposes in the case of retrospective studies (National Regulative Law number 12/2005 – I Serie-A, n°. 18–26th January, 2005).

Cell Culture

Human breast cancer cell line MCF-7/AZ was kindly provided by Prof. Marc Mareel (Ghent University, Belgium) [22], while BT-20 cells were purchased to American Type Culture Collection (ATCC, Manassas, VA). Cell lines were routinely maintained at 37°C, 5% CO_2, in the following media (Invitrogen): 50% DMEM/50% HamF12 (MCF-7/AZ), or only DMEM (BT-20). All media contained 10% of heat-inactivated foetal bovine serum (Greiner Bio-one, Wemmel, Belgium), 100 IU/mL penicillin and 100 mg/mL streptomycin (Invitrogen).

Transient Transfection

For gene reporter assays, cells were grown in 96-well plates to 60–70% confluence and transfection was done using the liposome-mediated FuGENE 6 transfection reagent (Roche Diagnostic GmbH, Mannheim, Germany), prepared according to the

manufacturer's instructions. A ratio of FuGENE/DNA of 3:1 was used. For protein expression assays, cells were grown in 6-well plates to 60% confluence. Transient transfections of C/EBPβ expression vectors were done using Lipofectamine 2000 (Invitrogen), with a ratio of Lipofectamine/DNA of 3:1 and prepared according to the manufacturer's instructions.

For knock-down assays, cells were transiently transfected at 60% confluence with specific siRNA for C/EBPβ (100 nM, FlexiTube siRNA – Hs_C/EBPβ 5-Qiagen) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's procedure. Maximum C/EBPβ knock-down was achieved after 48 h of incubation. A siRNA with no homology to any gene was also used as a negative control.

CDH3-luciferase Reporter Gene Analysis

Cells were co-transfected with pGL3-*CDH3*/luc promoter vector (20 ng) and with pRL-CMV Renilla vector (5 ng). For promoter analysis, 24 hours after transfection, cells were washed twice in PBS-cold and lysed for firefly/Renilla luciferase assays, using the Luclite Reporter Gene Assay System (Perkin Elmer), according to the manufacturer. Luciferase bioluminescence from Renilla was measured using native coelenterazine substrate reagent (Lux Biotechnology, Edinburgh, UK). Individual transfection experiments were repeated at least three times and in quadruplicate per transfection condition. Empty pGL3-basic vector and pGL3/luc-Control (pLUC) vector (Promega) were included as controls in all *CDH3*-reporter assays. Luminescence was read using the Wallac/Perkin Elmer-1450-028 Trilux Microbeta (Perkin Elmer) plate reader, and the results are shown as a mean of relative light units (RLU), which was calculated by the ratio between the luminescence signal emitted from luciferase and the luminescence signal obtained by the Renilla (normalization).

Western Blot

Cells were lysed and the concentration of total protein was determined by Bradford quantification. Western Blot was performed as earlier described [17,18]. For MCF-7/AZ cell line, due to its lower expression of P-cadherin, 50 μg of total protein lysate has been loaded; for BT-20, due to its P-cadherin overexpression, the gel loading was done only with 20 μg of protein lysate. Membranes were incubated with primary antibodies according to the conditions described in Table S1.

Site-Directed Mutagenesis

All the C/EBPβ binding sites mutations in *CDH3* promoter were performed in order to impair the binding of any predicted transcription factor: bioinformatic prediction tools were used to blast all point mutated sequences. To introduce point mutations in the *CDH3* promoter region, the QuickChange Site-directed Mutagenesis Protocol (Stratagene, Cedar Creek, USA) was followed, and the oligos used are listed in Table S2. The PCR cycles were set as follows: 95°C for 30 seconds; 16 cycles of 95°C for 30 seconds, 55°C for 1 minute, and 68°C for 5 minutes. Following PCR reaction, products were incubated with DpnI (1 hour at 37°C) and transformed into *E-coli* competent cells (Stratagene). All mutated plasmids were checked by sequencing and primer sequences are also listed in Table S2.

Chromatin Immunoprecipitation (ChIP) Assay

For chromatin immunoprecipitation of the endogenous *CDH3* promoter regions in MCF-7/AZ cells, the ChIP-IT^{TM} kit (Active Motif) was used and the assay was performed according with the manufacturer's procedures. Briefly, cells (4.5×10^7) were fixed with 1% formaldehyde in culture medium for 10 minutes. Fixation was stopped by incubating the cells for 5 minutes with a 1× Glycine Stop-Fix Solution, homogenized and centrifuged. The cell-pellets were resuspended in a shearing buffer and sonicated into chromatin fragments of 200–1500 bp in length. To reduce non-specific background, sonication-sheared lysates were pre-cleared with Protein G beads. The sheared chromatin lysates were incubated with 5 μg of C/EBPβ antibody or with a control rabbit IgG, overnight at 4°C, and immunoprecipitated with Protein G beads (2 hours at 4°C). The precipitated DNA-protein complex was washed 7 times, eluted, incubated for 8 hours at 65°C in a reverse cross-link buffer, and digested with proteinase K for 2 hours at 42°C. The resultant DNA was purified, resuspended in DEPC H_2O and quantified by real-time qPCR amplification. The PCR primers sequences used in this amplification are listed in Table S2.

For chromatin Immunoprecipitation in BT-20 cells and in an invasive breast carcinoma highly positive for P-cadherin and C/EBPβ, the Magna ChIP G Kit (Millipore) was used, according to manufacturer's protocol. Basically, the essential steps applied for BT-20 cells were the same as the ones used for MCF-7/AZ cells, differing only in the use of protein G magnetic beads instead of non-magnetic beads for simplicity of use. However, for the tumour sample, some alterations in the basic protocol were employed. Briefly, the tumour sample, that was frozen at −80°C since surgical extraction, was thawed and immediately fixed in 1% formaldehyde for 25 minutes, followed by the addition of 1× glycine solution for 5 minutes, washed in 1× PBS twice, frozen in liquid nitrogen, and finally pulverized mechanically. The following steps were the same used for breast cancer cell lines.

Statistical Analysis

Data are expressed as mean values of at least three independent experiments ± s.d. Student's t-tests were used to determine statistically significant differences ($*P<0.05$).

Results

P-cadherin is co-expressed with C/EBPβ and is regulated by this transcription factor in breast cancer cells

Using a large cohort of invasive breast carcinomas, the expression of C/EBPβ was previously demonstrated to be significantly associated with P-cadherin expression in about 60% of the cases [18]; however, the cellular co-expression of these two proteins was not verified. Thus, based on the hypothesis that C/EBPβ directly activates the *CDH3* gene promoter, a double immunostaining was performed in all invasive breast carcinomas that previously showed strong positivity for both proteins. As represented in Figure 1A, C/EBPβ expression was found in the nuclei of the same cells that were expressing P-cadherin at the cell membrane, pointing for a putative functional relationship between both proteins.

Based on these results, two different breast cancer cell models were used to demonstrate if P-cadherin expression could be affected by C/EBPβ: 1) MCF-7/AZ, which is an ER+/luminal type breast cancer cell line expressing moderate levels of P-cadherin, and 2) BT-20, an ER-negative/basal-like breast cancer cell line, highly positive for P-cadherin [17]. The siRNA mediated-knock-down of C/EBPβ induced a significant downregulation of all C/EBPβ isoforms (LAP1, LAP2 and LIP) in both cell lines. Interestingly, P-cadherin expression was also affected by the reduction of C/EBPβ isoforms, being this effect more pronounced in MCF-7/AZ cells (Figure 1B). According with these results, and

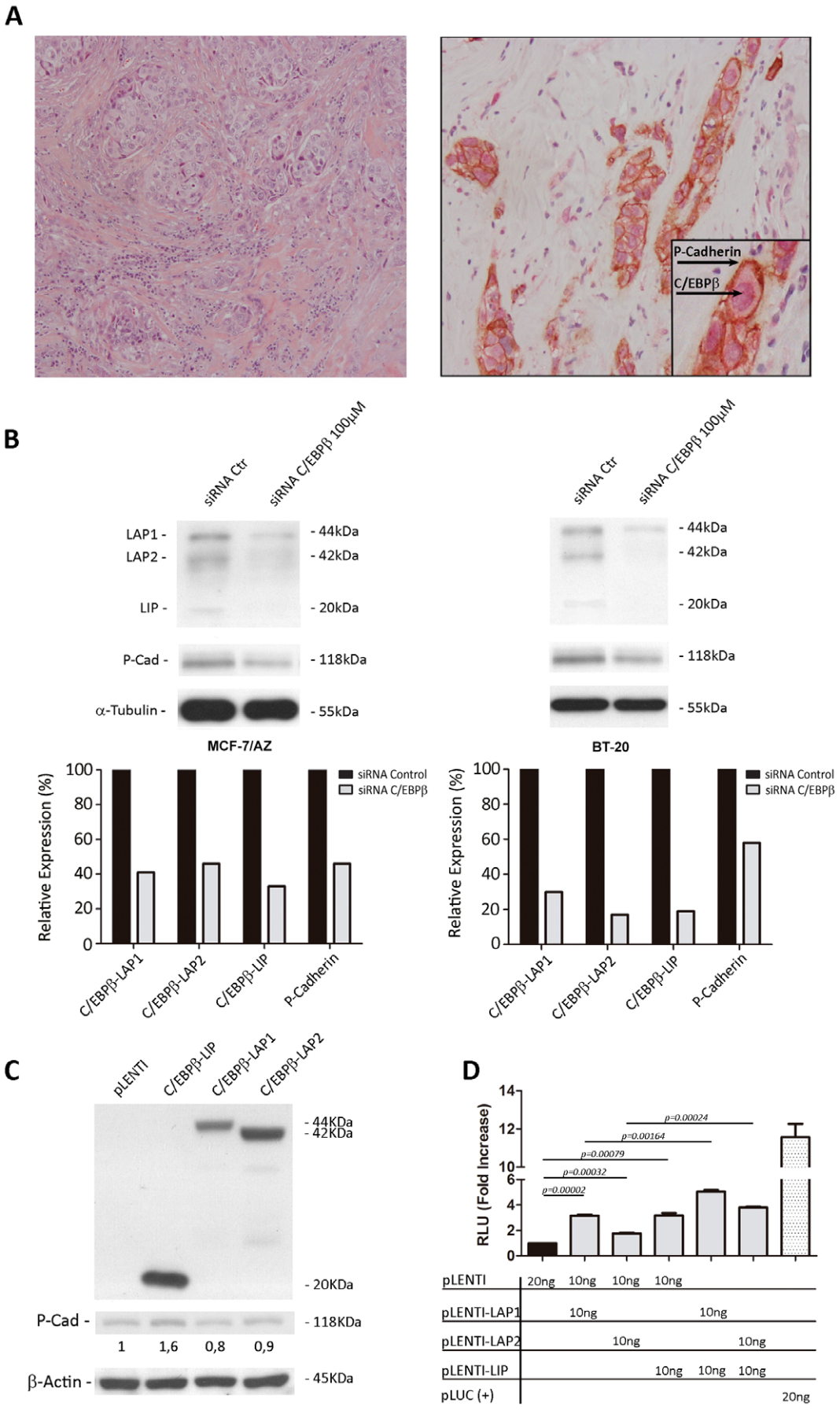
A
P-Cadherin
C/EBPβ
B
siRNA Ctr
siRNA C/EBPβ 100μM
LAP1 -
LAP2 -
LIP -
P-Cad -
α-Tubulin -
- 44kDa
- 42kDa
- 20kDa
- 118kDa
- 55kDa
MCF-7/AZ
BT-20
Relative Expression (%)
siRNA Control
siRNA C/EBPβ
C/EBPβ-LAP1
C/EBPβ-LAP2
C/EBPβ-LIP
P-Cadherin
C
pLENTI
C/EBPβ-LIP
C/EBPβ-LAP1
C/EBPβ-LAP2
- 44KDa
- 42KDa
- 20KDa
P-Cad -
- 118KDa
1
1,6
0,8
0,9
β-Actin -
- 45KDa
D
RLU (Fold Increase)
p=0.00024
p=0.00164
p=0.00079
p=0.00032
p=0.00002
pLENTI
pLENTI-LAP1
pLENTI-LAP2
pLENTI-LIP
pLUC (+)
20ng
10ng

Figure 1. Association and regulatory interplay between C/EBPβ and *CDH3*/P-cadherin expression in breast cancer cells. A) Double immunostaining for C/EBPβ and P-cadherin of an invasive breast carcinoma specimen (basal-like carcinoma, histological grade III), where it can be observed C/EBPβ expression in the nuclei and P-cadherin at the cell membrane of tumour cells (magnification ×200 and ×400-inset); a haematoxylin-eosin staining of this same case is shown to ascertain tissue integrity (magnification ×100); **B**) Using C/EBPβ-targeted siRNA, a consequent reduction of P-cadherin protein levels was observed in both MCF-7/AZ and BT-20 breast cancer cell lines; **C**) MCF-7/AZ cells transiently transfected with the different C/EBPβ isoforms (LAP1, LAP2 and LIP) displayed upregulation of P-cadherin protein levels only after induction of the C/EBPβ-LIP isoform; **D**) Luciferase reporter assays performed in cells transfected with the different C/EBPβ isoforms showed that the promoter activation induced by LIP and LAP1 isoforms was significantly greater compared with the activation induced by LAP2. The co-transfection of both LIP and each LAP1 or LAP2 induced the activation of the *CDH3* promoter in an additive manner.
doi:10.1371/journal.pone.0055749.g001

in order to decipher which C/EBPβ isoform was more relevant for P-cadherin activation, the expression of LAP1, LAP2 and LIP was induced in both breast cancer cell lines. As shown in Figure 1C, only C/EBPβ-LIP isoform was able to induce P-cadherin expression in more than 1.5-fold increase in MCF-7/AZ cells, while the remaining isoforms did not produce valuable effects on P-cadherin expression. This result was not found for BT-20 cells, probably due to their high basal levels of P-cadherin expression (data not shown).

Interestingly, in a previous study performed by our group, we found that the *CDH3*/P-cadherin promoter activation induced by the LIP isoform was significantly greater compared with the activation induced by LAP1 and LAP2 [18]. However, in the present study, this same experiment has been performed and, although the same significant result was observed at the promoter level for LIP ($p = 0.00079$), the *CDH3* promoter was also strongly and significantly activated by LAP1 ($p = 0.00002$) and less prominently, but also in a significant way, by LAP2 ($p = 0.00032$) (Figure 1D). Nevertheless, since it has been described that LIP can function as a dominant negative inhibitor of both LAP's activity [5], we decided to co-transfect both LIP and each LAP1 or LAP2 , in order to study their combined effect on *CDH3* promoter activity. The results showed that there is a significant increased activation of the promoter with any of the combinations compared with LAP1 or LAP2 alone, demonstrating that there is an additive effect of both isoforms ($p = 0.00164$ and $p = 0.00024$, respectively) on *CDH3* promoter activation, when added to LIP.

C/EBPβ physically interacts with endogenous *CDH3* gene promoter in breast cancer cells

Since the three C/EBPβ isoforms were able to transactivate the 1.8 Kb *CDH3* promoter gene construct (Figure 1D), we decided to evaluate in detail the sequence of this putative regulatory region using distinct bioinformatic tools, which can predict for the binding of specific transcription factors. Four concordant C/EBPβ-putative binding sites were identified within the first 1400 nucleotides. Interestingly, we found that there is a high degree of conservation of these predicted C/EBPβ binding sites between humans and other primates within the *CDH3* promoter (Figure 2A), and the left panel of Figure 2B shows their relative localization.

In fact, in order to demonstrate if there was a physical interaction between C/EBPβ proteins and *CDH3* promoter in these specific binding sites, ChIP has been performed in MCF-7/AZ breast cancer cells. Indeed, The results showed that there was an enrichment (relative to *input*) of the *CDH3* DNA-amplified fragments precipitated with the C/EBPβ antibody in all binding sites (Figure 2B, right panel), demonstrating that C/EBPβ transcription factors directly bind to the selected regions within the *CDH3* promoter.

This same experiment has been performed in BT-20 breast cancer cells, as well as in a frozen primary basal-like breast carcinoma, which was selected for being highly positive for P-cadherin and C/EBPβ expression. Interestingly, we could confirm the results, since there was precipitation with the C/EBPβ antibody in all the binding sites studied, in both cells and primary tumour (Figure 2C). Moreover, in BT-20 cells, which overexpress P-cadherin, the binding in all sites was very strong compared with the one found in MCF-7/AZ breast cancer cells.

C/EBPβ binding sites are important for *CDH3* gene activity and are selectively activated by the different C/EBPβ isoforms

In order to evaluate the importance of the aforementioned binding sites to the *CDH3* gene activation, as well as the specificity of the different C/EBPβ isoforms to the *CDH3* promoter, point mutations were introduced in the specific C/EBPβ binding sequences. Figure 3A illustrates the *CDH3* point mutations and their position within the C/EBPβ binding sites in relation to the wild-type *CDH3* promoter.

Interestingly, when MCF-7/AZ cells were transfected with the *CDH3* promoter containing point mutations at the binding sites 1 and 4 (*CDH3*-BS1 and BS4), there was a statistically significant alteration in *CDH3* promoter activity related to the wild-type promoter sequence (Figure 3B). In contrast, the activity of the *CDH3* promoter was not affected by the mutation introduced at the BS3 site, and only slightly affected by the introduced mutation at the binding site 2 (BS2). These results were mostly confirmed in BT-20 cells, especially for the BS4 mutation, located at the transcription start site region of the *CDH3* promoter, which also significantly induced its activity (Figure 3B). Although not significant, the reduction on *CDH3* promoter activity observed with the BS1 mutant was also found in BT-20 cells, suggesting that this distal C/EBPβ binding site is also important to *CDH3* gene transcriptional activation. In addition, the BS2 mutant significantly reduced *CDH3* promoter activity in BT-20 cells, showing that this is also a crucial site for the activation of P-cadherin transcription in this model. Finally, we could not find any effect of BS3 mutation in *CDH3* promoter activity also in BT-20 cells, proving that this site is not relevant for its regulation.

Since the distinct C/EBPβ isoforms have been documented has having different functions in cancer gene activation and in a cell-specific context, we co-transfected LAP1, LAP2 and LIP together with the different mutants of *CDH3* promoter in both breast cancer cell lines. The results demonstrated that distal *CDH3*-BS1 and BS2 are significantly important for the induced promoter activity mediated by all C/EBPβ isoforms. In contrast, BS3 did not play a significant role in C/EBPβ-mediated *CDH3* promoter activity, since mutations in this specific region were not important to impair the activation of *CDH3* gene mediated by any of the distinct isoforms. Similar results were observed concerning BS4, which did not reveal to be important for *CDH3* promoter activity mediated by LAP1, LAP2 or LIP isoforms. Finally, although not significant, the same trend was observed with BT-20 cells, proving that BS1 and BS2 are most likely the binding sites where all C/EBPβ isoforms bind to induce P-cadherin transcription in breast cancer.

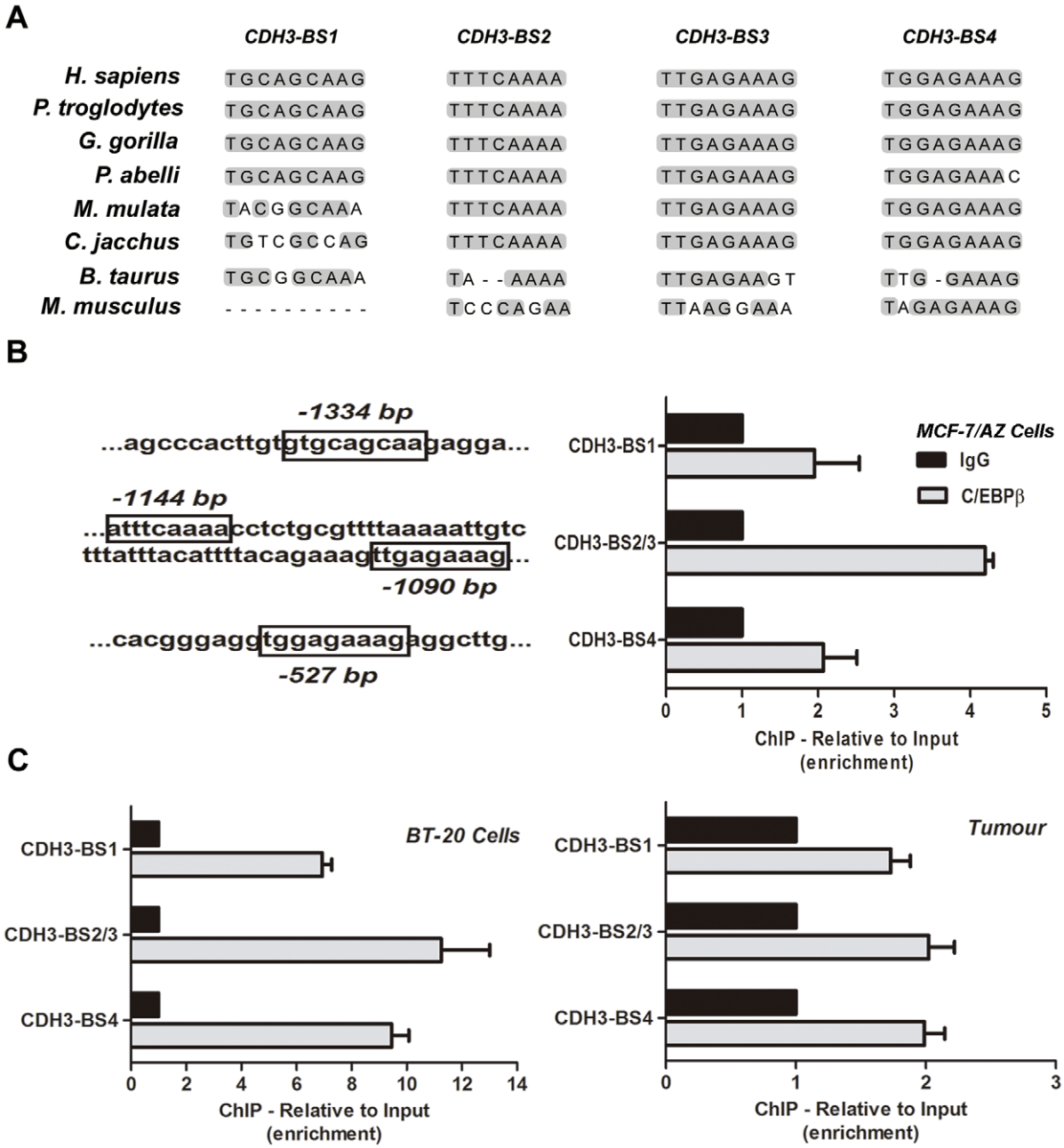

Figure 2. C/EBPβ physical interaction with the *CDH3* gene promoter. A) Putative C/EBPβ-binding sites within the *CDH3* gene promoter, where it can be observed their degree of conservation between human and other primates. Grey regions represent total sequence conservation in comparison with human sequence; **B**) Proximal regulatory region of *CDH3* promoter displaying the relative localization of the predicted C/EBPβ binding sites (left panel). The right panel illustrates the enrichment (relative to input) of the *CDH3* promoter DNA-amplified fragments precipitated from DNA-protein complexes obtained by ChIP in MCF-7/AZ breast cancer cells. **C**) ChIP experiment performed in BT-20 breast cancer cells and on a frozen primary breast tumour, highly positive for P-cadherin and C/EBPβ expression, also showed the same enrichment pattern for all the putative binding sites.
doi:10.1371/journal.pone.0055749.g002

Discussion

P-cadherin has been receiving a growing interest in the last years, since its overexpression is significantly associated with high histological grade breast tumours and with short-term patient overall survival [11,23–25]. The important association between P-cadherin expression and well-established markers correlated to breast cancer poor prognosis, such as high levels of Ki-67, epidermal growth factor receptor (EGFR), cytokeratin 5 (CK5), vimentin, p53 and HER2, has been also largely documented [11]. Although P-cadherin has been detected as altered in distinct tumour models, its effective role in the carcinogenesis process remains discussible, since it behaves differently depending on the studied cancer cell context [26]. If in some models P-cadherin has been suggested to act as an invasion suppressor, such as in colorectal cancer [27] or in melanoma [28], in several other models, including breast cancer, P-cadherin behaves as an

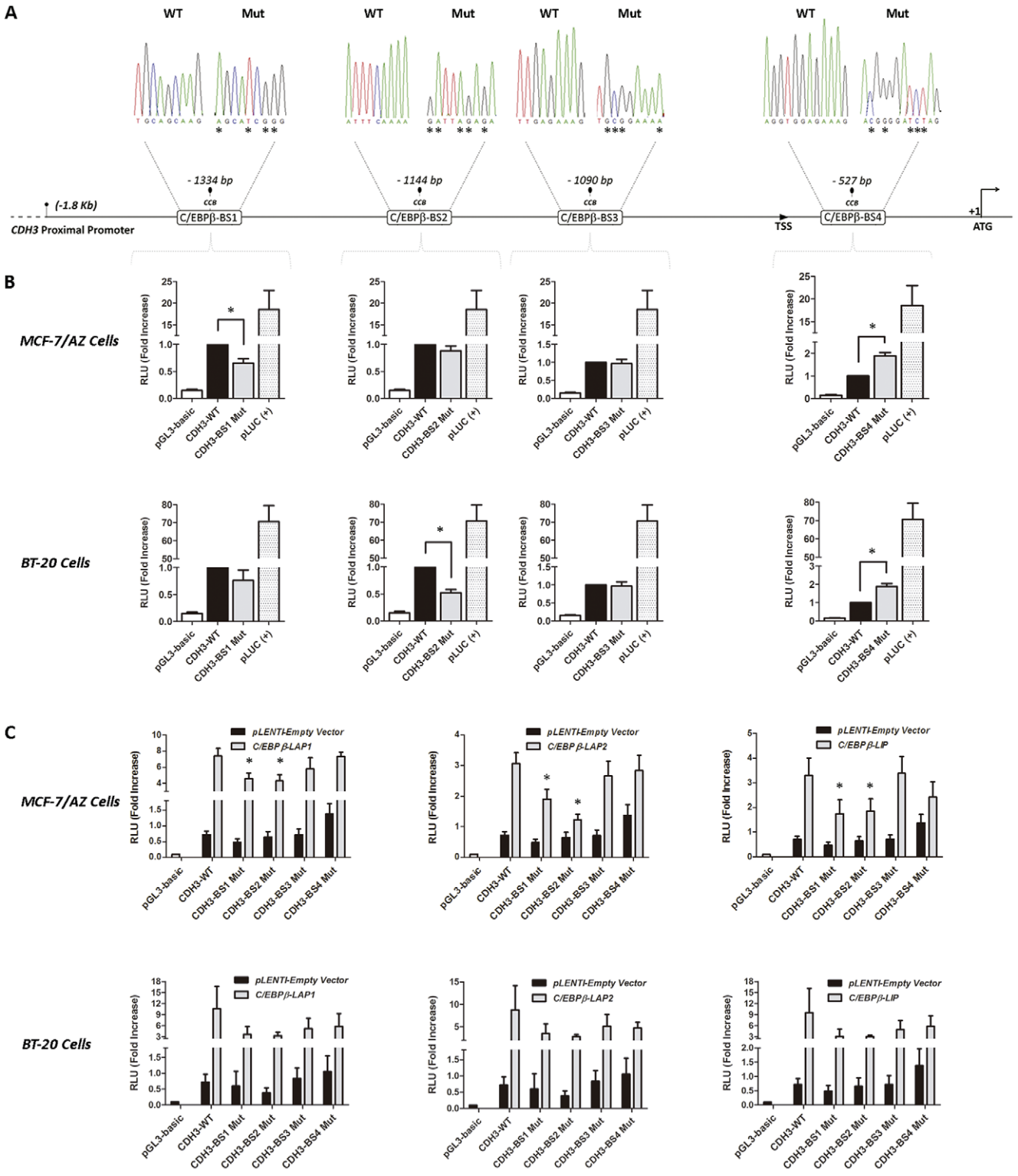

Figure 3. Relevance of C/EBPβ-isoforms and their putative binding sites in the activation of the *CDH3* gene. A) Schematic representation of the wild-type and mutated *CDH3* promoter; **B**) *CDH3*-Luciferase Reporter Assays performed with each of the mutations introduced at C/EBPβ binding sites demonstrating that *CDH3*-BS1, BS2 and BS4 are the most important for the activity of *CDH3* promoter in both MCF-7/AZ and BT-20 breast cancer cells; *p-value<0.05; **C**) *CDH3*-Luciferase Reporter Assays upon co-transfection of LAP1, LAP2 and LIP C/EBPβ isoforms, showing the relevance of specific C/EBPβ isoforms across *CDH3* promoter binding sites in both MCF-7/AZ and BT-20 breast cancer cells. *CDH3*-BS1 and BS2, but not BS3 and BS4, are responsive to all C/EBPβ isoforms; *p-value<0.05.
doi:10.1371/journal.pone.0055749.g003

oncogene, inducing increased tumour cell motility and invasiveness when aberrantly overexpressed [12–14,27,29–31].

However, data concerning *CDH3* gene regulation in breast cancer is still very limited. The induction of *CDH3* promoter activity in breast cancer cells was recently described by our group to be putatively linked to the transcription factor C/EBPβ, as well as P-cadherin and C/EBPβ expression have been reported to be highly associated in human breast carcinomas and linked with a worse prognosis of breast cancer patients [18]. In fact, the expression of C/EBPβ shares interesting biologic and functional features with the ones attributed to P-cadherin expression. Similarly to what has been described concerning C/EBPβ biology, P-cadherin is involved in homeostatic processes, such as cell differentiation, development and embryogenesis [32]. We have recently found that P-cadherin enriched cell populations show enhanced mammosphere forming efficiency (MFE), as well as increased expression of CD24, CD44 and CD49f, already described as normal or cancer stem cell markers. These results allowed to link P-cadherin expression to the luminal progenitor phenotype of the normal breast hierarchy and established an indirect effect of P-cadherin in stem cell biology [33]. Interestingly, these findings come along with observations that C/EBPβ regulates stem cell activity and specifies luminal cell fate in the mammary gland, categorizing C/EBPβ as one of the several critical transcription factors that specifies mammary stem cells fate during mammary gland development [34]. In a breast cancer biology setting, another interesting finding is related to the fact that P-cadherin, like C/EBPβ, is not mutated in breast tumours, but its overexpression has been widely described in a subset of aggressive breast cancers [5]. Importantly, at a clinicopathological level, some C/EBPβ isoforms, especially C/EBPβ-LIP, correlates with an ER-negative breast cancer phenotype, highly proliferative and high grade lesions and poor patient outcome [8,35]. All these characteristics overlap with the ones observed in highly malignant breast tumours overexpressing P-cadherin.

The present work demonstrates for the first time that P-cadherin and C/EBPβ co-localize in the same breast cancer cells, and that there is a physical interaction between this transcription factor and *CDH3* gene promoter. Herein, in addition to the identification of the promoter binding sites that are relevant for the transcriptional modulation of *CDH3* gene activity by C/EBPβ, we still tested the relevance of the different C/EBPβ isoforms along the *CDH3* promoter.

In fact, we show that C/EBPβ-LIP is the only isoform capable to significantly induce P-cadherin protein expression, confirming in a way the results obtained in our previous study, where a significant activation of the promoter was only revealed for LIP, although LAP1 and LAP2 were also able to activate the promoter. However, in this study, we found that *CDH3* gene is also significantly responsive to LAP1 and slightly to LAP2 isoform at the promoter level. These significant results were probably due to improved transfection efficiencies; however, although LAP1 and LAP2 are activating the gene promoter, supporting the classical knowledge described for these isoforms as transcriptional activators, this might not imply that these isoforms induce functional activity through protein synthesis. In fact, it has been largely discussed that the functionally transactivation potential of each C/EBPβ isoform can be highly modulated, since this ability strongly depends not only on dimer composition formed by C/EBPs, but specially on the partner proteins and responsive elements found in target gene promoters [5]. The fact that LIP activates *CDH3* promoter, leading to protein synthesis, reinforces the emerging evidence that LIP acts as a transcriptional activator of gene expression, challenging the long-standing concept that LIP fashionably functions as a dominant-negative isoform [5]. We also observed that LAP2 was the C/EBPβ isoform that activated *CDH3* promoter in a less extent, which is apparently surprising in light that LAP2 isoform is considered to be the most transcriptionally active C/EBPβ isoform [5]. However, it is also known that, in transformed cancer cells, an increase in LIP expression leads to a reduction in LAP2 activity and, therefore, impair its mediated transcription potential [36].

A novel observation also obtained in this study was the existence of interaction between C/EBPβ proteins to the conserved regions of the *CDH3* gene promoter, identified as C/EBPβ responsive elements. The ChIP results, obtained from the DNA region containing both BS2 and BS3 binding sites, revealed a cumulative increased C/EBPβ antibody-precipitated DNA when compared to individual BS1 and BS4, reinforcing the existence of bounding complexes. This was denoted for both MCF-7/AZ and BT-20 breast cancer cell lines and also for the basal-like tumour studied by *in vivo* ChIP.

Concerning the impact of C/EBPβ binding sites to the *CDH3* promoter activity, we found that BS1, BS2 and BS4 were the most relevant ones, while BS3 was not responsible for the modulation of the *CDH3* promoter. A detailed analysis of the *CDH3* promoter using the Ensemble ENCODE Project, revealed two DNAse Hypersensitive (DHS) sites located around BS1 and BS4 specific sequences, confirming an increased regulatory activity on these specific regions.

Interestingly, one of the most curious effects was the one found at BS4, which is located at the transcription start site region of *CDH3* promoter. In contrast with the distal sites, binding impairment at BS4 significantly induced the activity of *CDH3* promoter. In a first approach, we may hypothesize that specific C/EBPβ proteins are regulating negatively the activity of the promoter through that specific binding site and, upon mutation, this repression is released. However, since we did not find a significant effect mediated by LAP1, LAP2 or LIP when BS4 was mutated, we believe that other factors not C/EBPβ-related are responsible for the negative regulation in this binding site, or the mutation introduced in BS4 generated a sequence which allowed the binding of a transcription factor that is able to activate the *CDH3* gene promoter. Additionally, it is also interesting to note that, although the BS2 mutation did not create a significant decrease in *CDH3* promoter activity in MCF-7/AZ cells, this binding site is important to LAP2-mediated activation, indicating that it may not be endogenously active in these breast cancer cells, but probably highly active in BT-20 cells.

In conclusion, this study contributes to clarify the individual role of C/EBPβ proteins in breast cancer-related *CDH3*/P-cadherin gene, as well as to expand the limited characterization of the mechanisms and players that regulate this pro-invasive protein in breast cancer.

Supporting Information

Table S1 Conditions of the primary antibodies.
(PDF)

Table S2 Primers sequences used in the different assays.
(PDF)

Author Contributions

Conceived and designed the experiments: AA CR JP JCM RS FS. Performed the experiments: AA CR BS ARN ASR. Analyzed the data: AA JP FS. Contributed reagents/materials/analysis tools: AA CR JCM JP. Wrote the paper: AA JP FS.

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PUBLICATIONS

Paper 14

Cell Cycle 12:16, 2684–2690; August 15, 2013; © 2013 Landes Bioscience

Loss of caveolin-1 and gain of MCT4 expression in the tumor stroma

Key events in the progression from an in situ to an invasive breast carcinoma

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Keywords: DCIS, IDC, stroma, tumor progression, breast cancer, Caveolin-1, MCT4, immunohistochemistry

Abbreviations: DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma; Cav-1, caveolin-1; MCT4, monocarboxylate transporter 4; TMA, tissue microarray; H&E, hematoxylin-eosin; ER, estrogen receptor, PR, progesterone receptor; EGFR, epidermal growth factor receptor; CK, cytokeratin; P-cad, p-cadherin; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization

The progression from in situ to invasive breast carcinoma is still an event poorly understood. However, it has been suggested that interactions between the neoplastic cells and the tumor microenvironment may play an important role in this process. Thus, the determination of differential tumor-stromal metabolic interactions could be an important step in invasiveness.

The expression of stromal Caveolin-1 (Cav-1) has already been implicated in the progression from ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC). Additionally, stromal Cav-1 expression has been associated with the expression of stromal monocarboxylate transporter 4 (MCT4) in invasive breast cancer. However, the role of stromal MCT4 in invasiveness has never been explored, neither the association between Cav-1 and MCT4 in the transition from breast DCIS to IDC.

Therefore, our aim was to investigate in a series of breast cancer samples including matched in situ and invasive components, if there was a relationship between stromal Cav-1 and MCT4 in the progression from in situ to invasive carcinoma. We found loss of stromal Cav-1 in the progression to IDC in 75% of the cases. In contrast, MCT4 stromal expression was acquired in 87% of the IDCs. Interestingly, a concomitant loss of Cav-1 and gain of MCT4 was observed in the stroma of 75% of the cases, when matched in situ and invasive carcinomas were compared. These results suggest that alterations in Cav-1 and MCT4 may thus mark a critical point in the progression from in situ to invasive breast cancer.

Introduction

Breast cancer is a heterogeneous and complex disease, encompassing a variety of pathological entities with distinct clinical behaviors. The development of new technologies has offered the opportunity to explore the molecular complexity of human breast carcinomas.[1] However, despite these advances, the mechanisms controlling the transition from an in situ to an invasive carcinoma still remain unclear. Therefore, there is a significant interest in identifying molecular events driving invasive progression, not only to determine at which point the lesion is most likely to progress to malignancy, but also to identify new molecular targets that could trigger the progression at early stages.[1] Several studies have evaluated the gene expression profiles of both ductal carcinomas in situ (DCIS) and invasive ductal carcinomas (IDC),[2-8] but only few compared the in situ and invasive components within the same breast tumor.[5-8] In fact, although some genes have been described as differentially expressed between in situ and invasive components, the majority of the studies failed to demonstrate significant differences between the expression of the codified proteins in the neoplastic epithelial cells of DCIS and IDC.[5,9] Recently, our group, using patient-matched DCIS/IDC tumor samples, showed concordance between in situ and invasive molecular profiles in 94% of the cases.[10] These results suggested that the alterations in

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Submitted: 06/04/2013; Revised: 07/12/2013; Accepted: 07/17/2013
http://dx.doi.org/10.4161/cc.25794

the tumor microenvironment would have a more important role in the progression from an in situ to an invasive phenotype than the biology of the tumor cells per se, which showed a tendency to be maintained between these both components.

Actually, it is widely accepted that any cancer is a complex system composed not only by neoplastic cells but also by a fine-tuned microenvironment. The first reference to the importance of the microenvironment in cancer comes from Paget, with his proposal of the "seed and soil" hypothesis. Unexpectedly, this concept was "forgotten" and only "recovered" several years later. In breast cancer, tumor microenvironment plays a key role in defining tumor behavior and patient outcome.[11] Gene expression changes that occur in cancer-associated stroma are known to be implicated in prognosis, as well as in cancer progression.[12-14] Ma and colleagues, using gene expression profiling, provided strong evidence that the stroma co-evolves with the epithelial compartments during cancer progression.[12] Analyzing 14 patients with matched normal epithelium, normal stroma, tumor epithelium, and tumor-associated stroma, the authors proposed that microenvironment participates in tumorigenesis even before tumor cells invade the stroma, and it may play an important role in the transition from pre-invasive to invasive growth.[12]

Caveolin-1 (Cav-1), a scaffolding protein mainly involved in vesicular transport, cholesterol homeostasis, and signal transduction, has been associated to the progression from in situ to invasive carcinoma.[15,16] Lisanti and colleagues showed that Cav-1 loss in tumor stroma was associated with an increased risk for early recurrence, metastasis, and decreased overall survival in breast cancer, being also a strong prognostic factor for basal-like breast carcinomas.[17,18] In DCIS, a loss of stromal Cav-1 was predictive of disease recurrence and progression to invasive cancer, since all the patients with loss of Cav-1 recurred, and 80% of them progressed to invasive disease.[16] Moreover, loss of stromal Cav-1 has been related with stromal MCT4 expression in triple-negative breast cancers, also predicting for poor clinical outcome.[19] This protein is a major transporter directly responsible for L-lactate efflux from glycolytic cells and a functional marker of oxidative stress and hypoxia.[20] In addition, it seems to have a role in stromal breast cancer metabolism, since it has been demonstrated that breast cancer cells induce MCT4 overexpression in stromal fibroblasts.[21]

Since stromal expression of MCT4 and the association between Cav-1 and MCT4 had never been implicated in the progression from DCIS to IDC, the aim of this study was to better understand the stromal interactions surrounding in situ and invasive components of breast carcinomas, evaluating the stromal expression of Cav-1 and MCT4 using patient-matched DCIS/IDC tumor samples.

Results

IHC quantification for Cav-1 and MCT4 was performed on each set of the 22 TMA slides using patient-matched DCIS/IDC tumor samples. Data on ER, PgR, HER-2, P-cad, CK5, EGFR, Ki-67 status, histological grade, and lymph node metastases were already available and published for this series.[10]

Cav-1 and MCT4 expression in normal breast

In normal breast, it can be observed that Cav-1 expression was absent from the epithelium, whereas its expression was observed in the stromal component, as previously described.[16-18] MCT4 expression was absent in both epithelial and stromal components, as observed in **Figure 1A**.

Stromal Cav-1 expression in the progression from in situ to invasive carcinoma

In the DCIS component, only 19 cases (13%) showed no Cav-1 expression in the stroma, whereas 55 cases (39%) had moderate expression, and the majority had strong expression of stromal Cav-1 (67 cases, 48%). In the invasive component, the majority (n = 108, 76%) of the cases showed absent Cav-1 expression in the stroma, with only 27 cases (19%) with moderate expression and 7 cases (5%) with strong expression. **Figure 2** represents the expression levels of stromal Cav-1 in in situ and invasive components, where a significant decrease of Cav-1 from DCIS to IDC can be observed. An IHC example of Cav-1in in situ and invasive components is shown in **Figure 3**.

Regarding the progression from in situ to invasive carcinoma, analyzing each case for both matched components, 106 cases (75%) showed loss of stromal Cav-1 expression, whereas 35 (25%) cases maintained protein expression. None of the cases showed gain of stromal Cav-1 expression.

Stromal MCT4 expression in the progression from in situ to invasive carcinoma

Considering the DCIS component, the majority of the cases were negative (n = 131, 93%) (**Fig. 1B**), 10 cases (7%) showed moderate expression, and 5 cases (3%) were classified as strong for stromal MCT4. In the invasive component, a strong expression of MCT4 in the stroma of the majority of the cases (n = 73, 50%) was observed, whereas moderate expression was observed in 63 (43%) cases; in the remaining 11 cases (7%), no expression of stromal MCT4 was observed.

Figure 4 depicts the expression levels of stromal MCT4 in situ and invasive components, showing an increased expression of stromal MCT4 in the invasive component. **Figure 5** represents by IHC the strong MCT4 stromal expression in invasive component.

Concerning the transition from in situ to invasive carcinoma in terms of gains and losses of MCT4 in the stroma, we found that 126 cases (87%) gained expression in the invasive component, 19 cases (13%) maintained, and none lose the expression.

Combining stromal Cav-1/MCT4 in the progression from in situ to invasive carcinoma

Analyzing matched in situ and invasive components for stromal expression of Cav-1 and MCT4 (**Table 1**), it was possible to observe a statistically significant association between the loss of stromal Cav-1 and the concomitant gain of MCT4 in the same case ($P < 0.0001$). Interestingly, 75% of the cases that lost Cav-1 stromal expression in the transition from in situ to invasive cancer also gained MCT4 expression in the stroma. There were only 4 cases (3%) with loss of Cav-1 in the stroma that maintained MCT4 expression and 16 cases (12.5%) that gained MCT4 and maintained Cav-1 stromal expression. In 12 cases (10%), there was the maintenance of stromal expression for both

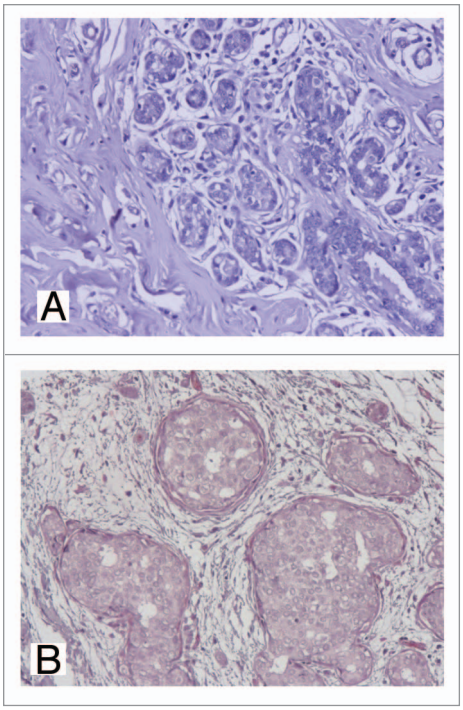

Figure 1. IHC expression of stromal MCT4 in normal and in situ component. Absent stromal MCT4 expression can be observed in normal breast (**A**) and in in situ component (**B**), 200×.

markers. **Figure 6** represents an IHC array with the expression levels of these proteins in the progression from in situ to invasive carcinoma.

Discussion

The mechanisms that mediate the progression from DCIS to IDC in the breast are still largely unknown. However, it is now widely acknowledged that accumulation of genetic anomalies contributes to the acquisition of an increasingly aggressive, invasive, or therapy-resistant tumor phenotype.[1] Nevertheless this knowledge did not improve the predictive power of standard pathological parameters for breast cancer, nor did it explain the mechanisms of invasiveness.

Cav-1 plays an important role in tumor stroma, and recent studies demonstrate that the loss of stromal Cav-1 is associated with advanced tumor and nodal stage, lymphovascular invasion, metastasis, early recurrence, tamoxifen resistance, and reduced progression-free survival in invasive breast cancer.[23-25] Additionally, loss of stromal Cav-1 also has prognostic value in a particularly aggressive subgroup of breast cancers, namely the triple-negative and basal-like breast carcinomas, whereas high levels of this protein were correlated with reduced tumor size, low grade, reduced metastasis, and improved survival.[18,25,26]

Interestingly, loss of stromal Cav-1 also predicts for recurrence and early disease progression in DCIS patients. Witkiewicz et al. reported that 80% of the DCIS patients, which underwent surgical excision and recurred with invasive breast cancers, showed reduced or absent levels of stromal Cav-1 in these tumors.[16] In

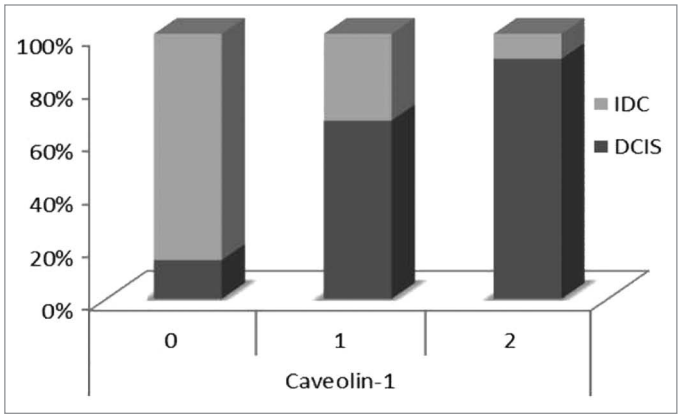

Figure 2. Expression levels of stromal Cav-1 in in situ and invasive components of breast carcinomas. It is possible to notice a significant decrease of Cav-1 stromal expression from DCIS to IDC.

our series, using patient-matched DCIS/IDC tumor samples, it was observed that the majority of the cases showed strong expression of Cav-1 expression in the stroma of DCIS, whereas 76% of the cases showed absent expression for this marker in the stroma of the invasive counterpart. Thus, regarding the progression to invasiveness, it seems that the loss of Cav-1 expression in the stroma is important for tumor invasion.

Actually, it has been already described that loss of Cav-1 in stromal cells may also increase angiogenesis and tumor growth.[15] Goetz et al. demonstrated that in vivo and in vitro expression of Cav-1 in cancer-associated fibroblasts facilitates tumor cells invasion and accelerates the in vitro proliferation and in vivo tumorigenesis.[27,28]

Recent data reveals that loss of Cav-1 induces a metabolic reprogramming of stromal cells to support the growth of adjacent epithelial tumor cells—the "reverse Warburg effect", where cancer cells induce upregulation of multiple glycolytic enzymes in neighboring stromal fibroblasts.[23,29,30] Cav-1 is degraded resulting in a loss of stromal Cav-1 expression.[19] At the same time, the breast cancer cells induce MCT4 overexpression in stromal fibroblasts.[19]

MCT4 is a monocarboxylate transporter that functions as a shuttle to extrude L-lactate from cells using aerobic glycolysis for energy metabolism.[20] Although the transporter role of MCT4 has been widely accepted in cancer epithelium, the prognostic value of MCT4 expression is highly compartment-specific and restricted to the tumor stroma, high stromal MCT4 levels being associated to poor patient overall survival.[21,31,32] In our series, analyzing DCIS and IDC separately, an increase of MCT4 expression was observed, since in DCIS the majority of the cases were negative, whereas, in the invasive counterpart, 50% of the cases showed strong expression for MCT4. Considering the progression from in situ to invasive breast carcinoma, using matched DCIS/IDC tumor samples, 87% cases gained MCT4 expression, whereas none showed loss of expression, suggesting that the gain of stroma MCT4 provides evidence for the existence of a stromal–epithelial lactate shuttle which fuels the tumor growth.[21]

Table 1. Association between stromal Cav-1 and MCT4 expression levels in the transition from in situ to invasive breast carcinoma

		MCT4 (in situ to invasive)		
		Loss of expression N (%)	Maintenance of expression N (%)	Gain of expression N (%)
Cav-1 (in situ to invasive)	Loss of expression N (%)	0 (0%)	4 (3%)	94 (75%)
	Maintenance of expression N (%)	0 (0%)	12 (10%)	16 (12.5%)
	Gain of expression N (%)	0 (0%)	0 (0%)	0 (0%)

P value ≤ 0.001

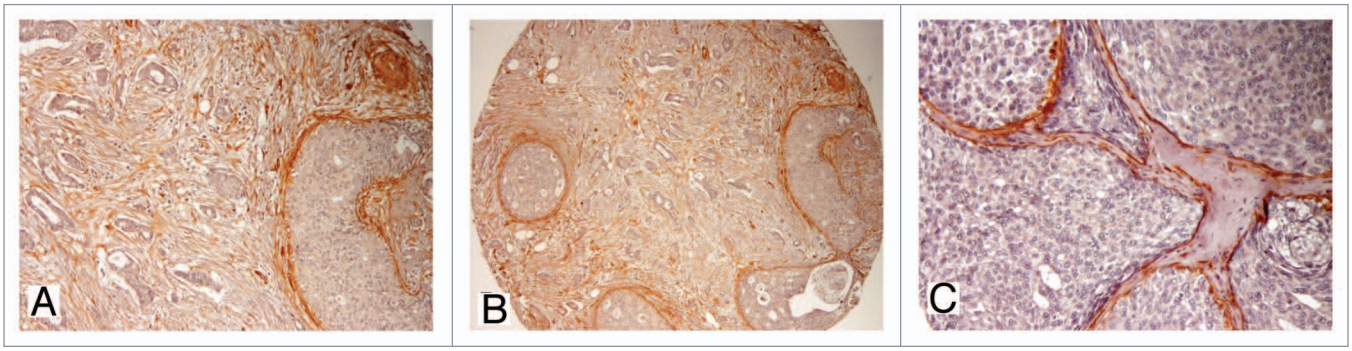

Figure 3. IHC expression of stromal Cav-1 in in situ and invasive components. Note the strong expression of Cav-1 in DCIS, from low (**A and B**, 100× and 200×, respectively) to higher magnification (**C**, 400×).

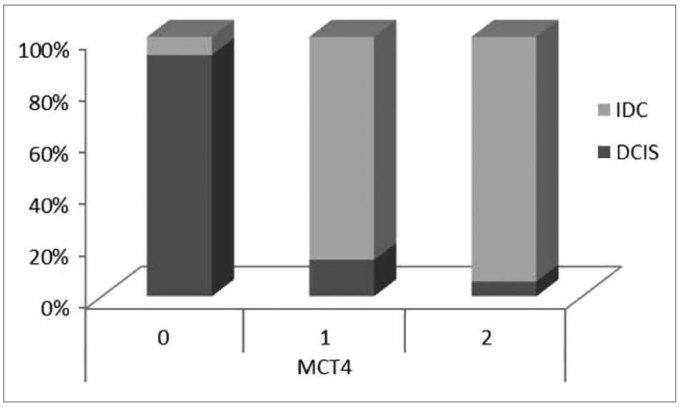

Figure 4. Expression levels of stromal MCT4 in in situ and invasive carcinomas. There is a significant increased expression of stromal MCT4 in the invasive component of breast carcinomas, when compared with DCIS.\

Regarding the relation between MCT4 and Cav-1 expression, Witkiewicz et al.,[19] using 164 invasive breast cancer samples, verified that stromal MCT4 and stromal Cav-1 levels were inversely related, high levels of stromal MCT4 being directly correlated with a loss of stromal Cav-1 immunostaining.[19] Most notably, cases with absent stromal Cav-1 are most likely to present strong stromal staining for MCT4, and, in contrast, cases with strong expression for Cav-1 are most likely to be stromal MCT4 absent.

Nevertheless, studies regarding the role of Cav-1 and MCT4 in the transition from in situ to invasive breast carcinoma were still lacking. In our series, using matched DCIS/IDC and analyzing the concomitant expression of stromal Cav-1 and MCT4, 75% of the cases showed loss of Cav-1 with simultaneous gain of MCT4 in the stroma, suggesting that these events are important for tumor cells to progress and invade.

Our results are explained by the recent "two-compartment tumor metabolism" model and the "reverse Warburg effect", suggesting that the loss of Cav-1 causes the metabolic reprogramming of stromal cells to support the growth of adjacent epithelial tumor cells.[23] In **Figure** 7, a hypothetical model summarizing he alterations in Cav-1 and MCT4 in the stroma of matched in situ and invasive breast carcinoma is shown.

The oxidative stress promoted by the tumor cells induces autophagy in cancer-associated fibroblasts (CAFS) that degrade Cav-1 in the in situ stromal compartment and also secrete energy-rich metabolites, such as L-lactate, ketone bodies, and pyruvate as a consequence of metabolic alterations. During the progression to invasive carcinoma, the loss of Cav-1 induces MCT4 expression due to the amount of energy metabolites, used to promote cancer cell glycolysis, aggressive tumor growth, and, ultimately, invasion of breast cancer cells.

Many of the cited studies quantify one or both markers in breast cancer stroma. However, one potential limitation of the quantification methodologies used is the lack of a clear and reproducible definition of stroma, especially regarding DCIS cases. In our case, since all IHC scoring was performed by the same experienced pathologist, we consider this does not affect internal validity and therefore does not affect the results obtained and conclusions drawn.

In summary, it was shown that the loss of stromal Cav-1 and the concomitant gain of stromal MCT4 have a putative role in

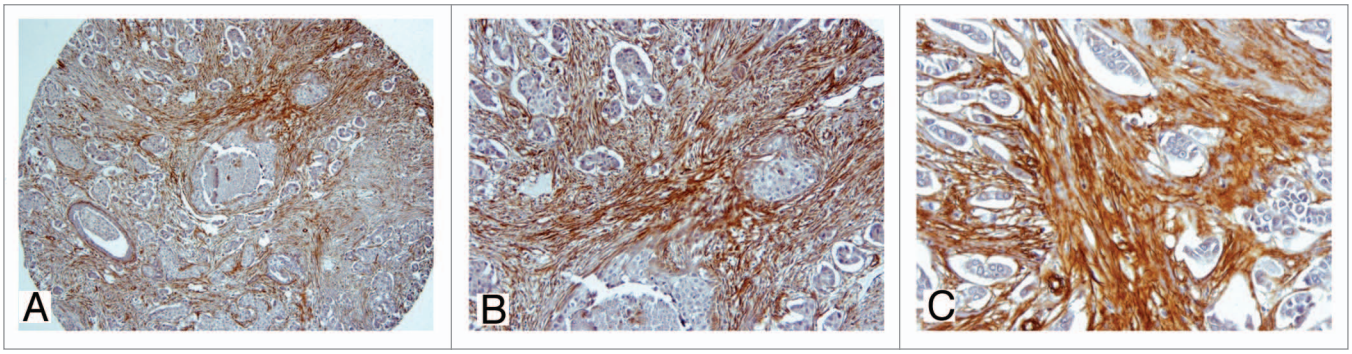

Figure 5. IHC expression of stromal MCT4 in in situ and invasive components. Note the strong MCT4 stromal expression in invasive component, from low (**A and B**, 100× and 200×, respectively) to high magnification (**C**, 400×).

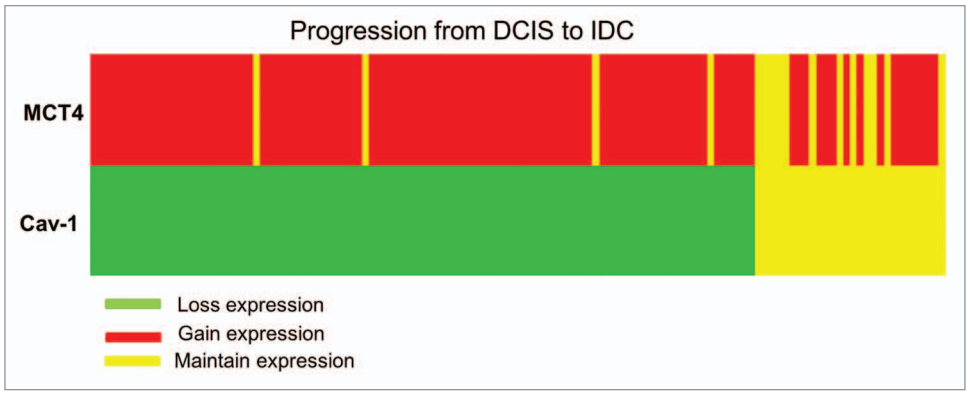

Figure 6. Immunohistochemistry array showing protein expression levels of stromal Cav-1 and MCT4 in the progression from in situ to invasive carcinomas. Cases are arranged along the *x*-axis and proteins are arranged along the *y*-axis. Within the heat map, red represents gain of expression, green represents loss of expression, and yellow represents maintained expression from in situ to invasive carcinoma within the same case.

the transition from in situ to invasive carcinoma of the breast. Therefore, we propose that Cav-1 and MCT4 may represent valuable biomarkers for breast cancer progression. Thus, determining the nature of the cooperation between tumor cells and the microenvironment that leads to invasion could identify therapeutic strategies to prevent the transition from in situ to invasive breast carcinoma.

Material and Methods

Case selection and TMA (tissue microarray) construction

Formalin-fixed and paraffin-embedded samples from 189 tumors, harboring in situ and invasive carcinoma areas in the same block, were consecutively retrieved from our archives. Available data included patient's age and clinicopathological features, such as tumor size and lymph nodes status. Representative areas of the in situ and invasive breast carcinomas were selected on H&E-stained sections and marked on the correspondent individual paraffin block. Two tissue cores (2 mm in diameter) were obtained from each specimen for TMA construction with each TMA block (donor block) and deposited into a paraffin block (receptor block) using a TMA workstation (TMA builder ab1802, Abcam). In each TMA block, non-neoplastic breast and liver tissue cores were also included as controls and TMA guide, respectively. An H&E-stained section from each TMA block was reviewed to confirm the presence of morphological representative areas of the original lesions.

All morphological and IHC assessments were conducted by a pathologist (FS). The study was conducted under the national regulative law for the handling of biological specimens from tumor banks, the samples being exclusively available for research purposes in retrospective studies.

Cav-1 and MCT4 immunohistochemistry

IHC was performed using the HRP labeled polymer (DakoCytomation) for Cav-1 and with the Ultravision Detection System Anti-polyvalent HRP (Lab Vision Corporation) for MCT4. Antigen unmasking was performed using a dilution of 1:100 from a commercially available solution of citrate buffer, pH = 6.0 (Vector Laboratories) at 98 °C. After the antigen retrieval procedure, the slides were washed in a phosphate buffer solution (PBS) and submitted to blockage of the endogenous peroxidase activity by incubation of the slides in a 3% hydrogen peroxide (Panreac) in methanol (Sigma-Aldrich). The slides were further incubated with the primary antibodies for Cav-1 (2297; BD Biosciences, diluted 1:50) and for MCT4 (H-90; Santa Cruz Biotechnology, diluted 1:500), as previously described.[24] All

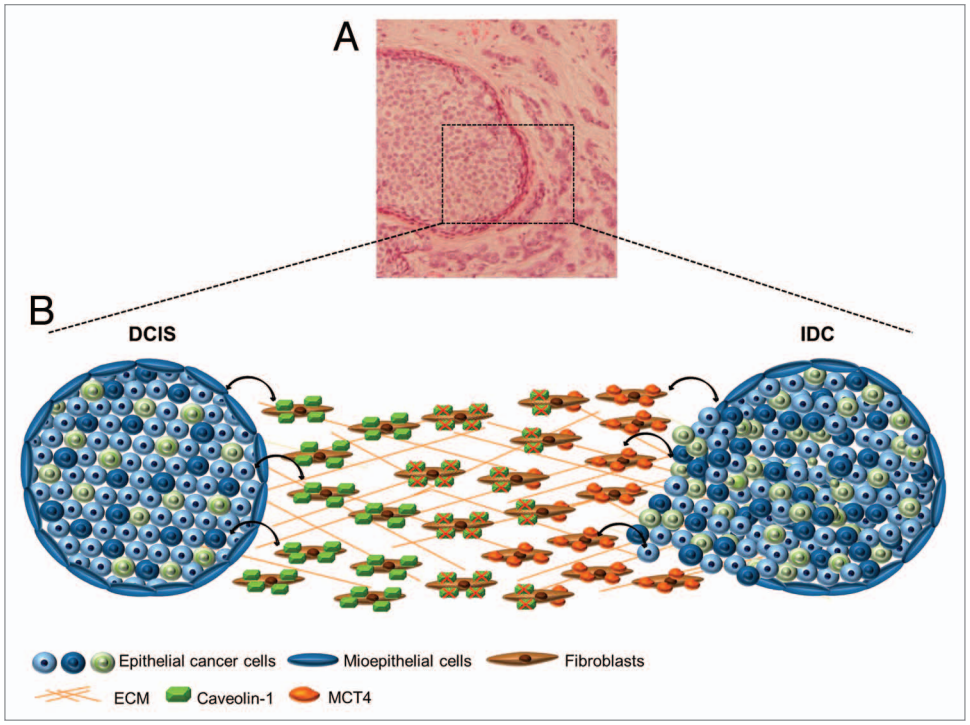

Figure 7. Alterations in Cav-1 and MCT4in the stroma of matched in situ and invasive breast carcinoma. (**A**) H&E-stained tissue section of human breast cancer, showing in situ and invasive components of breast carcinoma (100×). (**B**) Hypothetical model summarizing the importance of Cav-1 and MCT4 in the progression from DCIS to IDC. During the progression to invasive carcinoma, Cav-1 is degraded by oxidative stress-induced autophagy in cancer-associated fibroblasts, resulting in a loss of Cav-1. At the same time, the loss of Cav-1 induces a metabolic reprogramming of stromal cells, where cancer cells induce upregulation of MCT4 by stromal fibroblasts, in invasive counterpart.

reactions were revealed with diaminobenzidine (DAB) chromogen (DakoCytomation).

For both IHC assays, positive controls were included in each run, in order to guarantee the reliability of the assays. Non-neoplastic breast tissues, as well as normal breast surrounding the neoplastic cells, were considered internal controls.

Cav-1 and MCT4 immunohistochemistry evaluation

Cav-1 and MCT4 expression in stroma were evaluated using the previously described methodology.[16-19,21] In summary, Cav-1 and MCT4 were semi-quantitatively scored as negative (0, no staining), weak (1, either diffuse weak or strong staining in less than 30% of stromal cells per core), or strong (2, defined as strong staining in 30% or more of the stromal cells).[21]

Statistical analysis

Statistical analyses were conducted using StatView 5.0 software (SAS Institute Inc). The associations between categorical variables were tested for statistical significance using the chi-square test. A two-tailed significance level of 5% was considered as statistically significant ($P < 0.05$).

Disclose of Potential Conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

Martins D was involved in the construction and characterization of the breast cancer series used in the study and performed the majority of the experimental work and drafted the manuscript. Beça FF and Sousa B have made substantial contributions to the analysis and interpretation of the data. Baltazar F performed some of the immunoassays. Schmitt F was the pathologist that evaluated the immunohistochemical reactions. Paredes J and Schmitt F participated in the design of the study and its coordination and helped to draft the manuscript. All authors had final approval of the submitted and published versions.

Financial Disclosure Statement

This work was partially supported by research grants from Martins D (FCT-SFRH/BD/66152/2009); Sousa B (SFRH/BD/69353/2010); Paredes J (Ciência 2007: Contratação de Doutorados para o SCTN - financiamento pelo POPH - QREN - Tipologia 4.2 - Promoção do Emprego Científico, comparticipado pelo Fundo Social Europeu e por fundos nacionais do MCTES). IPATIMUP is an Associate Laboratory of the Portuguese Ministry of Science, Technology and Higher Education and is partially supported by FCT.

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PUBLICATIONS

Paper 15

P-cadherin signals through the laminin receptor α6β4 integrin to induce stem cell and invasive properties in basal-like breast cancer cells

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Keywords: P-cadherin, Breast cancer, α6β4 integrin, cancer stem cells, invasion, FAK, Src

***Received**: October 2, 2013* ***Accepted**: January 6, 2014* ***Published**: January 6, 2014*

ABSTRACT:

P-cadherin is a classical cell-cell adhesion molecule that, in contrast to E-cadherin, has a positive role in breast cancer progression, being considered a poor prognostic factor in this disease. In previous reports, we have shown that this protein induces cancer stem cell and invasive properties to basal-like breast cancer cells. Here, we clarify the downstream signaling pathways that are triggered by P-cadherin to mediate these effects.

We demonstrated that P-cadherin inhibition led to a significant decreased adhesion of cancer cells to the basement membrane substrate laminin, as well as to a major reduction in the expression of the laminin receptor α6β4 integrin. Remarkably, the expression of this heterodimer was required for the invasive capacity and increased mammosphere forming efficiency induced by P-cadherin expression. Moreover, we showed that P-cadherin transcriptionally up-regulates the α6 integrin subunit expression and directly interacts with the β4 integrin subunit. We still showed that P-cadherin downstream signaling, in response to laminin, involves the activation of focal adhesion (FAK), Src and AKT kinases. The association between the expression of P-cadherin, α6β4 heterodimer and the active FAK and Src phosphorylated forms was validated *in vivo*.

Our data establish that there is a crosstalk between P-cadherin and the laminin receptor α6β4 integrin signaling pathway, which link has never been previously described. The activation of this heterodimer explains the stem cell and invasive properties induced by P-cadherin to breast cancer cells, pointing to a new molecular mechanism that may be targeted to counteract the effects induced by this adhesion molecule.

INTRODUCTION

Cadherin molecules have a major role in tumor progression. A significant example is E-cadherin, for which a tumor suppressor function was already described in the majority of human cancer models. In fact, one of the first steps in the metastatic cascade is the loss or downregulation of E-cadherin expression or function by cancer cells, and it is known that mutations of its codifying gene (*CDH1*) increases the risk to develop particular types

of breast and gastric cancers [1]. P-cadherin, on the other hand, has a promoting effect in several solid tumors, including the ones from pancreas, prostate, colon and breast [1-6]. Indeed, we have previously demonstrated that P-cadherin is a poor prognostic factor for breast cancer patients, being significantly associated with lack of cell differentiation and high grade carcinomas [5, 7]. Its expression was found to be up-regulated in the particularly aggressive basal-like molecular subtype [5, 8, 9], and *in vitro* studies from our group have shown that P-cadherin increases cell invasion and motility [10], as well as induces the secretion and activation of metalloproteinases to the extracellular matrix (ECM) [11]. Recently, we further described its capacity to induce stem cell properties in basal-like breast cancer models [12].

The maintenance of stem cell activity requires signaling mediated by the ECM and by ECM receptors, also known as integrins [13]. Integrins play a major role in the integration of signals form the external microenvironment and from the cell internal milieu. In the normal breast, the basal/myoepithelial cells are in direct contact with the basement membrane, which is composed of a complex mixture of ECM molecules that contribute to the survival and adhesion signaling of epithelial cells and to the maintenance of the stem cell niche within this tissue. Interestingly, P-cadherin is also expressed by this basal cell layer and we have previously demonstrated that it is co-expressed with α6 integrin ECM-adhesion receptor (or CD49f) in a population of cells that show stem-like properties [12].

Alterations in the ECM or in the integrin expression are implicated in the initiation and progression of breast cancer [13, 14]. For example, ECM remodeling and integrin activation assist in the malignant transformation of cells in the primary site, as well as in the activation of quiescent cells in distant metastatic sites, such as the bone, liver, lung and brain [15-18]. In normal breast, the basement membrane has a crucial role in limiting tumor progression, being composed mainly by collagen type-IV and several laminins [19]; but, in cancer, elevated expression of laminin is considered a poor prognostic factor [19, 20]. In fact, abnormal overexpression of laminin-332 (formerly known as laminin 5) is present in the migrating edge of the tumor mass and the expression of laminin receptors are believed to promote invasion of breast cancer cells [19, 21]. Although several integrins recognize laminin substrates, the α6 integrins (α6β1 and α6β4) are the major receptors that contribute to breast cancer progression [22, 23]. Thus, the role of the heterodimer α6β4 in tumor progression has been extensively investigated. Aberrant activation of the α6β4 receptor is implicated in cell survival, migration and invasive potential [22-25]. Interestingly, the expression of the β4 integrin subunit is associated with poor breast cancer patient prognosis [20, 26] and specifically with the basal-like molecular subtype [26]. Although mice in which β4 integrin was inactivated in the mammary gland have a normal breast development [27], this integrin subunit was found to be crucial for breast cancer progression [28]. Furthermore, overexpression of the α6 integrin subunit was found in invasive breast carcinomas correlating with decreased overall patient survival [29], being an important breast stem cell marker in both mice and humans [30-33]. A major role has been also proposed for β1 integrin subunit in the normal development of the murine breast, being an important marker of normal murine stem cells [34] and regulating the ability of the stem cells to self-renew and properly differentiate during pregnancy [35]. This integrin molecule has also an important role in tumorigenesis, since the disruption of this integrin in the mammary gland of a polyomavirus middle T antigen (PyMT) transgenic mouse model completely blocked tumor formation [36].

Thus, the crosstalk between cell-cell and cell-ECM adhesion complexes reflects a highly integrated intracellular network. Integrin and cadherin adhesion molecules often cooperate, activating the same signaling pathways and eliciting similar cellular functions that are part of a larger adhesive structure. In cancer, an association of cadherins and integrins can originate complexes that mediate important oncogenic responses, often through interaction with other transmembrane proteins, such as growth factor receptors. Several reports focus on the association of E-cadherin with integrin molecules [37-40], but no interaction between P-cadherin and integrin molecules has ever been described. P-cadherin is well described as having a role in cell-cell interactions; however, its role in cell-ECM interaction remains completely unknown.

The aim of this study was to find out if the P-cadherin-induced aggressive features were dependent on microenvironment signals, in particular, the ECM components and integrin receptors. Herewith, we demonstrated that P-cadherin is needed for breast cancer cell adhesion to specific ECM components. The expression of the laminin receptor α6β4 integrin was found to depend on P-cadherin expression. Moreover, this integrin heterodimer was involved in the mammosphere formation ability induced by P-cadherin expression in breast cancer cells. A new signaling mechanism triggered by P-cadherin is described that involves the activation of FAK, Src and AKT kinases in response to laminin.

RESULTS

P-cadherin expression potentiates the adhesion of basal-like breast cancer cells to laminin

The role of P-cadherin as a cell-cell adhesion molecule is well documented; however, its role in cell-ECM adhesion is completely unknown. Thus, the adhesion

of cancer cells to several ECM components typically implicated in tumour progression was assessed.

The basal-like epithelial breast cancer cell lines MDA-MD-468 and BT-20 were used as model systems, which are characterized by the positive expression of E-cadherin, negativity for hormone-receptors, lack of HER-2 amplification and high levels of basal markers, including high expression of EGFR and P-cadherin. Transient knock-down of P-cadherin was performed by siRNA (60% inhibition in MDA-MB-468 and 82% inhibition in BT-20, at the protein level) and adhesion to collagen type-I, collagen type-IV, laminin-332, vitronectin and fibronectin was measured by the crystal-violet adhesion assay (Figure 1).

MDA-MB-468 and BT-20 control cells preferentially adhered to collagen type-I and vitronectin, followed by a moderate adhesion to collagen type-IV, laminin-332 and fibronectin. Adhesion of both cell lines to plastic was approximately 70% for MDA-MB-468 cells and 40% for BT-20 cells (Figure 1A). When P-cadherin was inhibited in MDA-MB-468 cells, adhesion to laminin-332, vitronectin and fibronectin was significantly reduced by about 20%, whereas adhesion to the collagen molecules (type I and IV) was not affected (Figure 1A and 1B). For the BT-20 cell line, a significant 25% decrease in the adhesion to the laminin substrate was specifically observed after P-cadherin knock-down (Figure 1A).

P-cadherin regulates the expression of the laminin receptor α6β4 integrin in breast cancer cells

Since P-cadherin regulates the adhesion of cancer cells to specific ECM components, we set out to investigate whether this effect was mediated by any alteration in the expression of integrins, the main receptors involved in ECM-cell adhesion. β1 integrin is a major component of most integrin heterodimers, recognizing several ECM components, including laminin, vitronectin and fibronectin. Vitronectin and fibronectin are also recognized by RGD integrin receptors, specifically containing β3, αV and α5 integrin subunits [41]. Laminin is mainly recognized by integrins that contain α6 and β4 subunits, which bind exclusively to this ECM substrate, and it is described as having important tumor promoting effects in breast cancer [22-25]. Based on this knowledge, we analyzed the surface expression of β1, β3, β4, α5, α6, and αV integrin subunits by flow cytometry in the MDA-MB-468 and BT-20 basal-like breast cancer models, with or without the silencing of P-cadherin transcripts by siRNA.

As shown in Figure 2, the inhibition of P-cadherin

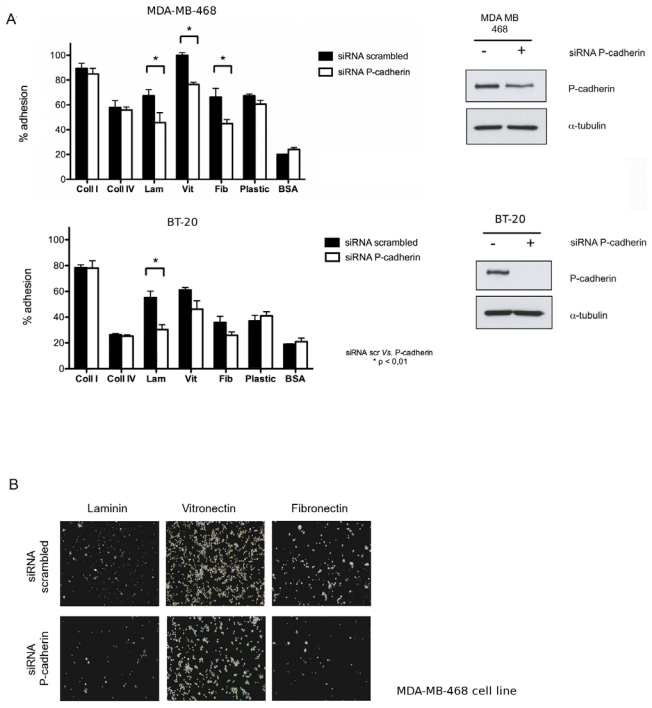

Figure 1: Adhesion of basal-like breast cancer cell lines to extracellular matrix (ECM) components is dependent on P-cadherin expression. (A) Inhibition of P-cadherin expression in MDA-MB-468 cells decreased % adhesion to laminin-332, vitronectin and fibronectin (adhesion time = 20 min). A significant decrease in cell adhesion was also observed for BT-20 cell line in laminin (adhesion time = 30 min); (B) Bright field images of MDA-MB-468 cells in the tissue culture plate coated with ECM substrates after the adhesion assay. Cells were fixed and the nuclei stained with crystal-violet. (Coll I – collagen I, Coll IV – collagen IV, Lam – laminin 332, Vit – vitronectin, Fib – fibronectin, BSA – bovine serum albumin, negative control).

expression had no effect in the expression of β1 or β3 integrins in both cell lines analyzed. Despite having found a reduction in the α5 and αV integrin subunits in MDA-MB-468 cells, no differences were found in the cell surface expression of these integrins in BT-20. Noteworthy, P-cadherin knock-down caused a reduction in the cell surface expression of the α6 and β4 integrin subunits in both cell lines (Figure 2A). Interestingly, α6 and β4 form a heterodimer (also known as hemidesmosome in normal cells) that recognizes the major component of the basement membrane, laminin-332, for which we demonstrated that adhesion was impaired upon P-cadherin knock-down in both cell lines analyzed (Figure 1A).

Furthermore, the expression of α6 and β4 subunits was also evaluated by immunofluorescence and immunoblotting, confirming a decrease in the total amount of these integrins in breast cancer cells after P-cadherin knock-down (Figure 3A and Supplementary Figure 1). The decrease in the α6 integrin protein expression is accompanied by a decrease in the mRNA levels of the α6 integrin/*ITGA6* gene, whereas the mRNA levels of the β4 integrin/*ITGB4* gene are not affected (Figure 3B). Since lateral integrin-cadherin associations are known to occur [37, 39, 42], we also tested the existence of a physical interaction between P-cadherin and α6β4 integrin; the β4 integrin subunit and P-cadherin were able to co-immunoprecipitate (Figure 3C).

P-cadherin and the α6 integrin confer stem cell properties and invasive features to breast cancer cells

Since P-cadherin expression impacts cell-ECM adhesion and clearly modifies integrin α6β4 expression in breast cancer cells, we set out to study if this integrin heterodimer was also implicated in the aggressive properties that have been previously ascribed to P-cadherin, namely, the invasive capacity and the mammosphere forming ability. Furthermore, to clarify the crosstalk between P-cadherin and α6β4 integrin, the effect of both α6 and β4 integrin subunits in the expression levels of P-cadherin were also studied.

Inhibition of α6β4 in breast cancer cells significantly decreased the mammosphere forming efficiency (MFE), as well as the invasion capacity, precisely in the same magnitude as the one induced by P-cadherin inhibition (Figure 4A and 4B). Importantly, α6 integrin inhibition alone showed the same impact in MFE and in the invasion potential as the inhibition of P-cadherin or the

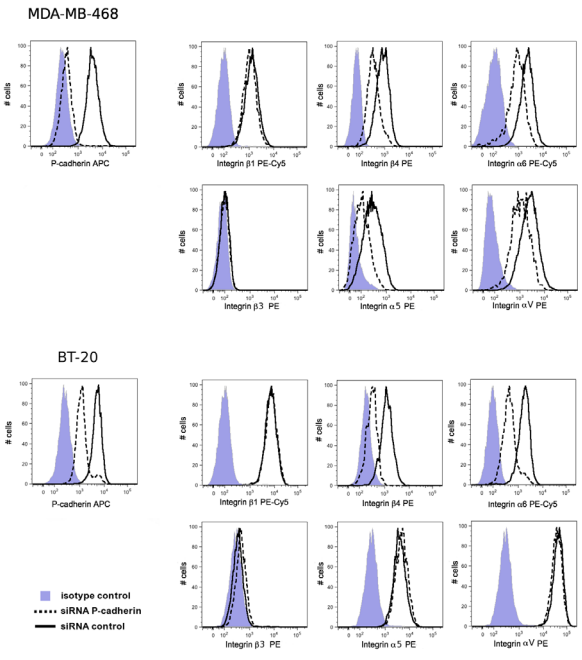

Figure 2: P-cadherin knock-down reduces integrin α6 and β4 expression in MDA-MB-468 and in BT-20 cells. Cell surface expression of P-cadherin and integrin molecules was analyzed by flow cytometry. The median intensity of integrins α6 and β4 stain was decreased upon P-cadherin knock-down. No effect was observed in the expression of the integrin subunits β1 or β3.

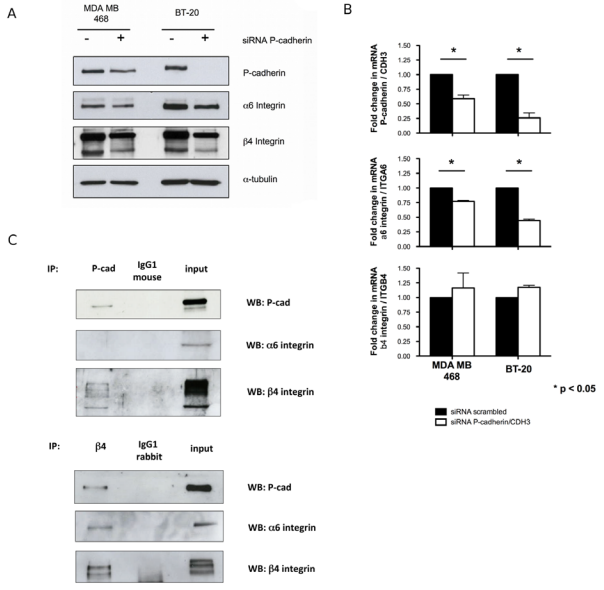

Figure 3: P-cadherin controls the expression of α6β4 integrin heterodimer in basal-like breast cancer cell lines. Transient inhibition of the P-cadherin/CDH3 gene in MDA-MB-468 and BT-20 cells leads to a decrease in the expression of α6 and β4 integrin subunits, measured by western blot (A); the mRNA level of α6 integrin/ITGA6 is decreased upon P-cadherin silencing, whereas β4 integrin/ITGB4 mRNA level is unaffected (B); Co-immunoprecipitation experiments show that P-cadherin directly interacts with the β4 integrin subunit, but not with the α6 integrin subunit in these cells (the BT-20 cell line is represented).

repression of the α6β4 heterodimer. However, inhibition of the β4 integrin subunit in breast cancer cells did not show a statistically significant impact in these functional properties (Figure 4A and 4B). These results indicate that P-cadherin downstream signaling effects could be primarily dependent on the α6 integrin subunit function.

It is also interesting to note that, while P-cadherin knock-down caused a reduction in α6 and β4 integrin subunits, the opposite was not true (Figure 4C). The inhibition of α6 and/or β4 integrins showed no effect in P-cadherin expression. Nonetheless, α6 integrin knock-down led to a decrease in the expression of its partner, the β4 integrin subunit, pointing that P-cadherin may in fact be controlling the α6 subunit expression, which in turn controls the β4 subunit, as already shown by Klinowska and colleagues [27]. In summary, the functional properties attributed to P-cadherin expression were only affected when the α6 integrin subunit or the α6β4 integrin heterodimer were inhibited; the inhibition of β4 integrin subunit had no effect in MFE and invasion.

P-cadherin overexpressing cells have increased adhesion to laminin as well as increased mammosphere forming ability and these properties are dependent on α6β4 integrin expression

The previous results indicated that there could be a crosstalk between two adhesion molecules: P-cadherin and α6 integrin. Thus, to further explore the role of α6 integrin and its partner, β4 integrin, in the functional properties mediated by P-cadherin, we analyzed the cell-laminin adhesion capacity and the MFE of a breast cancer cell line constitutively overexpressing P-cadherin (MCF7/AZ.P-cad) and compared these properties with control cells, which have low levels of P-cadherin (MCF7/AZ.mock). P-cadherin expression was accompanied by an increase in the expression of both, the α6 integrin subunit, as well as the β4 integrin subunit (Figure 5A). Importantly, P-cadherin upregulation led to an increase in the adhesion of MCF7/AZ.P-cad cells on top of a laminin coated

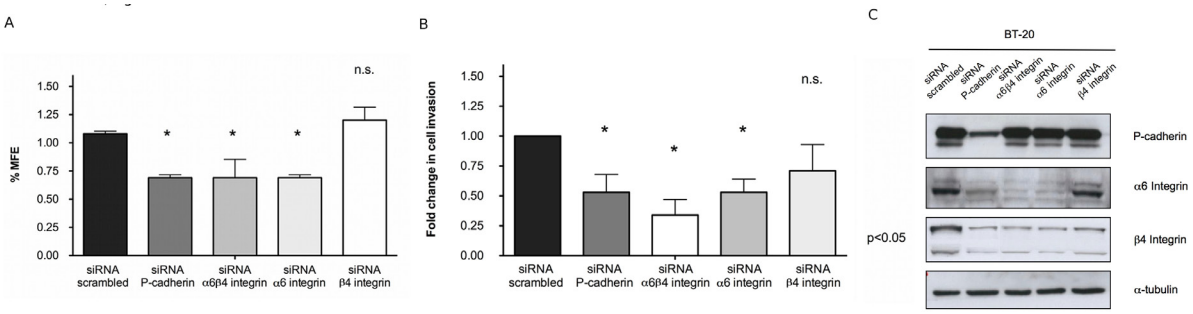

Figure 4: Inhibition of α6β4 integrin decreases the mammosphere forming efficiency (MFE) in breast cancer cells to the same extent as inhibition of P-cadherin (A). In the same way, the invasion capacity of these cells in matrigel was severely compromised when P-cadherin or α6β4 integrin were knocked-down (B); although the expression of the α6 and β4 subunits is decreased upon P-cadherin knock-down, the expression of P-cadherin is not affected after the inhibition of either α6 integrin or β4 integrin or both integrins at the same time (C). Results for the BT-20 cell line are shown in the figure. Similar results were observed for the other basal-like cell line, MDA-MB-468.

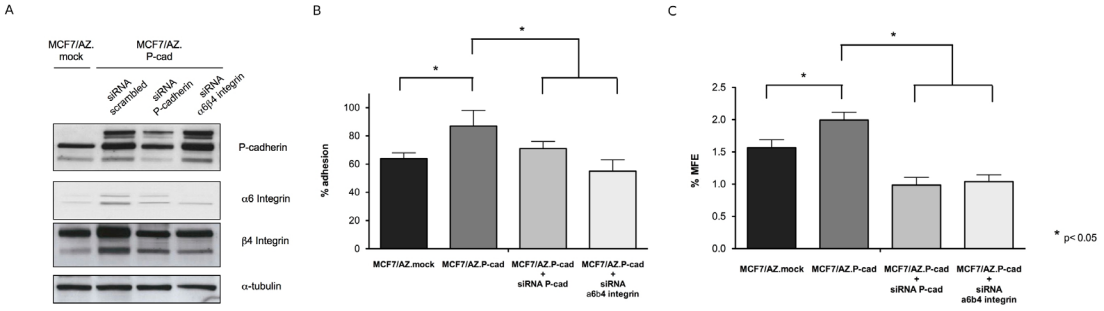

Figure 5: P-cadherin overexpression induces increased adhesion to laminin-332 and increased mammosphere forming capacity in a α6β4 integrin dependent manner. P-cadherin overexpression in MCF7/AZ cells (MCF7/AZ.P-cad) induces the expression of α6 and β4 integrin subunits (Vs. MCF7/AZ.mock cells), measured by western-blot (A); P-cadherin overexpression also induced increased adhesion to laminin-332, evaluated by the adhesion assay (adhesion time = 30min) (B) and increased mammosphere forming efficiency (MFE) (C). Inhibition of the α6β4 integrin heterodimer in the P-cadherin overexpressing cells restored the levels of adhesion to the control levels, and strongly inhibited the MFE in these cells. P-cadherin expression was unaffected by α6β4 integrin knock down.

surface (Figure 5B) and increased the mammosphere forming ability of these cells (Figure 5C). These effects were mediated, at least partially, by α6β4 integrin expression, since the levels of this integrin heterodimer are increased in P-cadherin overexpressing cells (Figure 5A); when both integrin subunits were simultaneously knocked-down in MCF7/AZ.P-cad cells, these functional properties were significantly reduced (Figure 5). Once more, P-cadherin levels were not affected by α6β4 integrin knock down, indicating that these integrin molecules are most likely acting downstream of P-cadherin activation (Figure 5A).

Integrin signaling in response to laminin-332 is dependent on P-cadherin expression

The previous data established a cross-talk between P-cadherin and α6β4 integrin in basal-like breast cancer cell models. We therefore studied whether P-cadherin could affect the main signaling molecules downstream of the α6β4 integrin receptor in cancer cells, when these were grown on top of a laminin substrate. The activation of the integrin related kinases FAK and Src was studied by immunoblotting after cell adhesion to this substrate. We found that P-cadherin inhibition in breast cancer cells reduced p-FAK Tyr397 and p-Src Tyr416 levels (Figure 6A). Notably, the p-FAK Tyr397 reduction was also detected by immunofluorescence in both cell lines studied (Figure 6B). Furthermore, activation of AKT was also affected, shown by a reduction in level of p-AKT Ser473 (Figure 6A). Altogether, these results indicate that FAK and Src activation in response to laminin is dependent on P-cadherin expression in basal-like breast cancer cells.

We also investigated if the cancer cell phenotype was affected in cells grown on top of the substrate for α6β4 integrin. Thus, we analyzed the cytoskeleton microfilaments by phalloidin staining by fluorescence microscopy in breast cancer cells adhered to laminin coated coverslips (Figure 6B). We found that control cells (scrambled transfected) had more stress fibres and appeared more flattened than cancer cells with P-cadherin

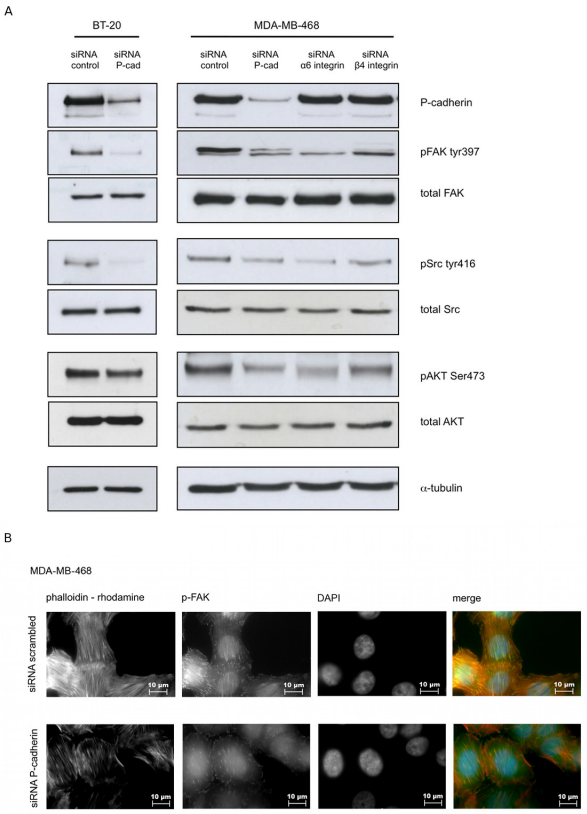

Figure 6: P-cadherin signaling in response to laminin involves FAK/Src activation. Analysis of integrin downstream signaling molecules in breast cancer cell lines was performed after adhesion to laminin-332 (20 min for MDA-MB-468 and 30 min for BT-20) (A); The number of stress fibers (F-actin was stained with phalloidin-rhodamine) and focal adhesions/contacts (stained with pFAK Tyr397 – Alexa 488) is reduced by P-cadherin knockdown in MDAB-MB-468 cells grown on top of laminin (B). The same result was found for BT-20 cell line.

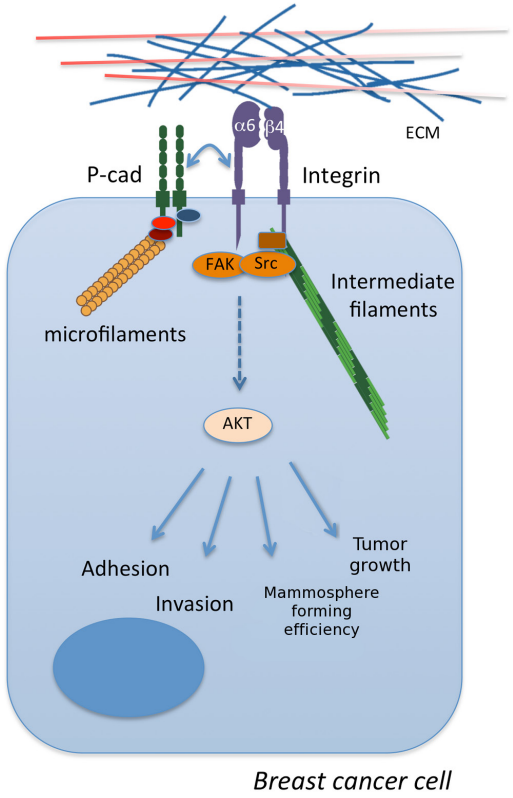

Figure 7: The crosstalk between P-cadherin and α6β4 integrin in basal-like breast cancer. These signaling molecules cooperate leading to the phosphorylation and activation of FAK, Src and AKT, mediating tumor growth and important aggressive cancer properties *in vitro,* such as cell invasion, adhesion to laminin and mammosphere formation.

Table 1: *In vivo* association of P-cadherin expression with the α6β4 integrin heterodimer and the activation of FAK/Src pathway. Tumours were formed *in vivo* from the MDA-MB-468 cell line with different levels of P-cadherin expression and they were characterized by IHC for the expression of α6 integrin, β4 integrin, pFAK and pSrc. A statistically significant association was found between P-cadherin and the expression of α6 integrin, β4 integrin, pFAK and pSrc (Fisher's exact test)

		P-cadherin		
		High (n=4)	Low (n=12)	p value
α6 integrin	Positive (n=5)	4 (100%)	1 (8.33%)	0.0027
	Negative/low (n=11)	0 (0%)	11 (91.67%)	
β4 integrin	positive (n=5)	4 (100%)	1 (n=8.33%)	0.0027
	Negative/low (n=11)	0 (0%)	11 (n=91.67%)	
pFAK	Positive (n=3)	3 (75%)	0 (0%)	0.0071
	Negative/low (n=13)	1 (25%)	12 (n=100%)	
pSrc	Positive (n=)	4 (100%)	3 (25%)	0.0192
	Negative/low (n=)	0 (0%)	9 (75%)	

knock-down. The stress fibres provide the cytoskeletal tension which is required for focal adhesion formation in laminin, indicating a strong adhesion to the ECM substrate. Staining with an antibody for p-FAK Tyr397 allowed the identification of focal adhesions and sites of cell-to-cell contacts. Both focal adhesions and cell-cell contacts were decreased in P-cadherin depleted cells (Figure 6B). In summary, P-cadherin has a role in eliciting cell shape changes associated with adhesion to the ECM.

Assessment of P-cadherin/α6β4 integrin/FAK/Src cross-talk signaling in in vivo tumor xenografts

Since the data collected *in vitro* pointed out to an activation of FAK/Src signaling in a P-cadherin dependent manner, we decided to analyze if this signaling pathway was also present in the *in vivo* setting. In order to study tumors with different P-cadherin expression levels, we have used the basal-like and P-cadherin positive MDA-MB-468 cell line and FACS to separate the top 20% P-cadherin expressing cells from the low 20% P-cadherin expressing cells (purity of sorted populations was 85-95%). These sorted cells, as well as the unsorted population, were inoculated into the subcutaneous region, under the left abdominal mammary fat pad of immune compromised mice. The tumorigenic capacity was evaluated after 30 days and tumors were characterized by immunohistochemistry for P-cadherin, α6 integrin, β4 integrin, pFAK and pSrc.

The percentage of tumors formed with the unsorted population was 66.6% (8/12 mice). The top 20% P-cadherin group of animals presented an increased tumor formation capacity (85.7%, 6/7 mice) compared to the P-cadherin low 20% group (28.6%, 2/7 mice). All the tumors formed were histologically classified as solid with infiltrative growth and extensive necrosis (Supplementary Figure 2A).

Concerning the immunohistochemical analysis, 25% (4/16) of the tumors showed high expression for P-cadherin, 31.2% (5/16) of the tumors formed were considered positive for α6 and β4 integrins, 18.7% (3/16) were positive for pFAK and 43.7% (7/16) were positive for pSrc (Supplementary Figure 2 and Supplementary Table).

Our results showed that there is a statistically significant association between the tumors with a high expression of P-cadherin (P-cad high) and the expression of α6 integrin (p=0.0027), β4 integrin (p=0.0027), pFAK (p=0.0071) and pSrc (p=0.0192) (Table 1), validating the signaling pathway previously found *in vitro*. Additionally, we were also able to find an association between α6 integrin and β4 integrin (p=0.0005, data not shown), as well as between pFAK and both integrin subunits (p=0.0179, data not shown).

Discussion

Cadherins are classically seen as molecules that make a major contribution for cell-to-cell adhesion. Specifically in breast, P-cadherin expression is found in the myoepithelial cell layer, strongly contributing to the self-organization of these cells [43]. Notably, this basal layer of the mammary epithelium is also enriched in molecules involved in the adhesion of epithelial cells to the ECM, namely in integrin molecules, such as α6β1 and α6β4. In normal cells, the later heterodimer is known as hemidesmosome and it is the receptor for laminin, the major component of the basement membrane.

In breast cancer, P-cadherin molecule appears upregulated in 30-40% of all diagnosed cases, being

significantly associated with poor patient prognosis [5, 44]. It is known, however, that breast cancer progression involves modifications of the normal ECM, as well as oncogenic activation of integrin signaling in both primary tumors, as well as in the metastatic sites [16, 45]. Here, we established that P-cadherin is involved in the attachment of cells to ECM substrates, since its silencing rendered cancer cells significantly less able to adhere to vitronectin, fibronectin and laminin. When integrins expression was investigated, we found that P-cadherin was necessary for the appropriate expression of the integrin subunits α6 and β4. Importantly, the recognition of laminin by cancer cells has significant tumor promoting effects. For example, laminin-332 induces motility in the MCF-7 breast cancer cell line [46]. Furthermore, IHC analysis of laminin-332 in human *in situ* breast carcinomas showed that this ECM substrate is located in the myoepithelium adjacent to preinvasive cells [21, 46], that could potentially contribute to the early steps of stromal invasion. The interface zone between the tumour cells and the stroma is enriched in laminin, as well as in α6β4 integrin [21].

We have previously shown that P-cadherin induces invasion and migration of breast cancer cells [11] and plays an important role in breast tumourigenesis *in vivo* [12, 47]. The signaling pathways that contribute to this aggressive behavior are poorly understood, involving to some extent the activation of metalloproteinases and the consequent release of a soluble pro-invasive P-cadherin fragment and/or the activation of small GTPases [6, 11]. Recently, we have shown that P-cadherin has been implicated in the maintenance of stem and progenitor properties in basal-like breast cancer cells, including the self-renewal capacity and the tumorigenic ability in nude mice [12]. We also found that P-cadherin is co-expressed with α6 integrin in breast cancer cells [12], a marker of the stem/progenitor phenotype present in the mouse and human breast [30-33]. In the present work, we explored further this association, showing that there is a crosstalk between both adhesion molecules. P-cadherin is acting upstream of a major signaling pathway that involves the activation of α6 integrin and its partner, the β4 subunit. As a consequence of the adhesion of cancer cells to laminin surface, the activation of the α6β4 heterodimer would lead to Src and FAK activation in a P-cadherin dependent manner. In fact, in the present study, we showed that P-cadherin knock-down reduces FAK and Src phosphorylation *in vitro* and an association was found between P-cadherin with the α6β4 heterodimer and FAK/Src activation *in vivo*. Importantly, it has been reported that α6β4 integrin promotes survival and invasion by activating the PI3K/Akt pathway [24, 48] and notably, in our work, a reduction was also found in AKT activation in P-cadherin silenced cells.

It was previously found that α6 integrin activation induces P-cadherin transcription [49], further supporting the idea that P-cadherin could cooperate with α6 integrin signaling. However, our work revealed that P-cadherin and α6 integrin do not directly interact. Rather, P-cadherin expression seems to control the transcription of α6 integrin subunit, since silencing of P-cadherin leads to a decrease in the α6 integrin mRNA levels. On the other hand, β4 integrin mRNA levels were unaffected by P-cadherin inhibition, but the physical interaction between P-cadherin and β4 integrin subunit points to a possible regulation at a post-transcriptional level. In fact, the absence of β4 integrin after P-cadherin silencing may be due to the downregulation of its unique partner, the α6 integrin, hence blocking the formation of the heterodimer, as already shown by Klinowska and colleagues [27].

β1 integrin is also a partner of α6 integrin subunit recognizing laminin, being also essential for the correct development of the mammary epithelium, and regulating the ability of the mammary stem cells to self-renew and differentiate properly [35]. Despite not having found any alteration in the β1 integrin levels upon P-cadherin inhibition, we do not exclude the possibility that β1 integrin subunit is also implicated in the maintenance/acquisition of cancer stem cell and invasive properties, as this is the other major partner of α6 integrin, constituting an important laminin receptor.

Additionally, although the cell morphology was not severely affected by P-cadherin knock-down and cells clearly maintained an epithelial phenotype, we found that the number of cell-to-cell contacts and the number of focal adhesions to laminin was clearly reduced upon P-cadherin inhibition. It is possible that α6β4 integrin, and the subsequent FAK/Src kinase activity, may also be contributing to the stem/progenitor characteristics. It was shown that FAK deletion in the murine mammary gland suppressed tumorigenesis by decreasing the number of cancer stem cells ($CD24^+CD29^+CD61^+$ and $ALDEFLUOR^+$ populations) [50]. Notably, FAK activation allows for the survival of cells in anchorage-independent conditions [51], which may explain why integrin knock-down, as well as P-cadherin knock-down, reduced survival of cells growing as suspension colonies in which the ECM is present within the mammosphere.

Thus, the poor patient prognosis found in P-cadherin overexpressing breast cancer cases [10] may be related, at least partially, to the fact that this cadherin enables cells to respond to integrin signaling, promoting an oncogenic response. Importantly, strategies to inhibit P-cadherin could lead to a decrease in integrin activation and potentially oppose the oncogenic signaling mediated by laminin and its receptor. Since P-cadherin up-regulation is also found in *in situ* stages of breast cancer development [7], it is possible that it may already be contributing to the changes in integrin signaling in the early stages of breast cancer development.

In conclusion, our results show that P-cadherin controls the cell-to-laminin adhesion, by modulating the expression and the activation of the α6β4 integrin

heterodimer. Moreover, P-cadherin and α6β4 integrin have oncogenic signaling pathways that cooperate and cross-talk, in order to induce cancer cell invasion and survival in anchorage independent conditions. These results are particularly relevant, since they provide a new link for P-cadherin and the tumor microenvironment and a new molecular mechanism explaining P-cadherin aggressive behavior in breast carcinomas.

METHODS

Ethics statement

Investigation has been conducted in accordance with the ethical standards and according to the Declaration of Helsinki and according to national and international guidelines and has been approved by the authors' institutional review board.

Cell culture

Human breast cancer cell lines MDA-MB-468 and BT-20 were obtained from ATCC (American Type Culture Collection, Manassas, VA). These cell lines were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic solution (penicillin–streptomycin) (Invitrogen, Carlsbad, CA). The human breast cancer cell line MCF-7/AZ was obtained from a collection developed in the laboratory of Prof. Marc Mareel (Ghent University Hospital, Belgium), which was genetically manipulated to stably overexpress P-cadherin (MCF-7/AZ.Pcad). The control cell line (MCF-7/AZ.mock) shows low P-cadherin levels, identical to the parental cell line [10]. These cell lines were cultured in DMEM/F12 supplemented with 10% FBS and 1% antibiotic solution (penicillin–streptomycin) (Invitrogen). All cells were routinely cultured in a humidified atmosphere with 5% CO_2 and at 37ºC and were used in experiments when reached 70–80% confluence.

RNA knock-down of P-cadherin and integrin molecules

Gene silencing was conducted by siRNA sequences targeting specific genes. P-cadherin (*CDH3* gene) target sequence: AAGCCTCTTACCTGCCGTAAA, Integrin □6 (*ITGA6* gene) target sequence: CAGGGTAATAAACTTAGGTAA, Integrin □4 (*ITGB4* gene) target sequence: GTGGATGAGTTCCGGAATAAA. All siRNA sequences were obtained from Qiagen (Hilden, Germany). Cell transfection was carried out using HiPerFect transfection reagent (Qiagen) in a final concentration of 5 nM siRNA, according to the manufacturer's instructions. Optimal silencing of the target genes was achieved at 48hafter transfection, which was confirmed by immunoblot analysis. A siRNA scrambled sequence was included as a control (Qiagen).

Adhesion assay to ECM substrates

Cell adhesion assay was performed by the crystal violet assay in 96-well microtiter plates coated with laminin-332 (Sigma, St. Louis, MO), fibronectin (Sigma), vitronectin (BD Biosciences, San Diego, CA), type-I or type-IV collagen (Sigma) (5μg/ml) overnight at 4ºC. Subsequently, plates were washed three times in PBS and non-specific-binding sites were blocked by adding 0.5% BSA (w/v) in PBS containing Pen/Strep (Invitrogen) for 2h at 37ºC. Once washed again with PBS, 100μl of cells (10^6 cells/ml) were seeded in serum-free medium for 20 minutes (for MDA-MB-468 cell line) or 30 minutes (for BT-20 cell line). Thereafter, the plates were washed with PBS to remove non-adherent cells, and the attached cells were fixed with acetone:methanol (1:1) for 10 minutes at 4ºC. Cell adhesion was determined following the colorimetric method described by Busk [52]. The absorbance was measured at 570nm with a microplate reader. The attachment of cells to wells coated with 1mg/ml of poly-L-Lys (Sigma) and fixed with 4% paraformaldehyde before aspiration was defined as 100% of adhesion.

Flow Cytometry analysis

Cells were harvested with versene/0.48mM EDTA (Invitrogen). Detached cells were washed with PBS supplemented with 0.5% FBS and re-suspended in the stain buffer (2mM EDTA + 0.5% bovine albumin in PBS). A single cell suspension was labeled by fluorescence-conjugated antibodies at a concentration of 1 to 10 in stain buffer: PE-Cy5-conjugated Integrin β1 (CD29), PE-Cy5-conjugated Integrin α6 (CD49f) or PE-conjugated Integrin β4 (CD104). These antibodies were obtained from BD Biosciences (San Diego, CA). P-cadherin monoclonal antibody APC-conjugated was obtained from R&D (Minneapolis, MN) and used at the same concentration as above. A live-dead stain (Invitrogen) and the primary antibodies or the respective isotype controls (BD Biosciences) were incubated at 4°C, in the dark, for 15 minutes. The labeled cells were then washed in the stain buffer and analyzed on a LSR-II or FACS Canto-II (BD Biosciences).

Immunofluorescence microscopy

Different cell lines were seeded on top of glass coverslips coated with laminin-332 (Sigma). Cells were

fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and blocked with 5% BSA before staining. The following primary antibodies were used for immunofluorescence: FITC-conjugated α6 integrin (CD49f) (1:10, BD Biosciences), PE-conjugated β4 integrin (1:10, CD104) (BD Biosciences) and p-FAK tyr397 (1:200 dilution, Cell Signaling). To visualize p-FAK, anti-rabbit Alexa-488 (1:1000, Invitrogen) was incubated on slides for 30 minutes. F-actin was detected by staining with phalloidin conjugated to rhodamine (Invitrogen) at a dilution of 1:1000. Cells were visualised using a Zeiss Imager Z.1 microscope (Zeiss, Welwyn Garden City, UK) and representative photos were acquired using the associated software: Photoshop and Illustrator (both CS4; Adobe).

Immunoblotting analysis

After performing the adhesion assay over a laminin coated surface (6 wells plate, BD Biosciences), cells were lysed with PBS containing 1% Nonidet-P40 (NP40, Sigma-Aldrich, St. Louis, MO) and phosphatase (Sigma) and protease inhibitors (Roche Diagnostics Gmbh, Mannheim, Germany). Protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Richmond, CA) and 30 μg of total protein was resolved on a 10% denaturing polyacrylamide gel and transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway NJ). After blocking nonspecific binding with 5% non-fat dry milk (for non-phosphorylated protein detection) or 5% BSA (for phosphorylated protein detection) in PBS containing 0.5% Tween 20, each membrane was incubated for 1 hour at room temperature with each of the following primary antibodies: anti-P-cadherin (1:500, clone 56, BD Transduction), anti-α6 integrin (1:1000, Sigma-Aldrich), anti-β4 integrin (1:2000, Santa Cruz Biotechnology), anti-pSrc Tyr 416 (1:1000, Cell Signalling, Danver, MA), anti-total Src (1:1000, Cell Signalling), anti-pFAK Tyr 397 (1:1000, Cell Signalling), anti-total FAK (1:500, BD Transduction), anti-pAKT Ser 473 (1:2000, Cell Signalling) and anti-AKT1/2 (1:500, Santa Cruz Biotechnology). Anti-α-tubulin (1:10000, clone DM1A, Sigma-Aldrich) was used in all the blots as a loading control. Secondary antibodies were peroxidase-conjugated from Santa Cruz Biotechnology. Immunoreactive proteins were detected by enhanced chemiluminescence detection kit (Amersham, GE Healthcare, Uppsala, Sweeden) and exposure to Hiperfilm ECL (Amersham).

Mammosphere assay

Monolayer cells were enzymatically detached with 0.125% trypsin-EDTA (Sigma-Aldrich), manually disaggregated with a 25-gauge needle to a single-cell suspension and suspended in cold PBS. Cells were plated at 500/cm^2 in non-adherent culture conditions, in flasks coated with 1.2% poly-2-hydroxyethylmethacrylate / 95% ethanol (Sigma). Cells were grown, for 5 days, in DMEM/F12 containing B27 supplement, 500 ng/ml hydrocortisone, 40 ng/ml insulin, 20 ng/ml EGF and maintained in a humidified incubator at 37°C and 5% (v/v) CO_2. Mammosphere forming efficiency (MFE) was calculated as the number of mammospheres (≥50 μm) formed divided by the total number of cells initially plated, being expressed as a percentage.

Invasion assay

Matrigel invasion assay was performed according to manufacturer's instructions (BD Biosciences). Briefly, transwell chambers with polycarbonate membrane filters (6.5 mm diameter, 8 μm pore size) were coated with 20 μL of a Matrigel solution. 3.5x10^4 BT-20 cells or MCF7/AZ cells were added to the upper compartment of the chamber. The lower compartment was filled with DMEM medium supplemented with 10% FBS and 1% antibiotic solution (penicillin–streptomycin) (Invitrogen). After 24 or 48 hours of incubation (BT20 or MCF7/AZ, respectively) at 37°C, 5% CO_2, the upper surface of the filter was washed with serum-free DMEM and cleared from non-migratory cells with a cotton swab. The remaining (invasive) cells at the lower surface of the filter were fixed with cold methanol and stained with 4′, 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, 0.4 mg/mL). Invasive cells were scored by counting the whole filter with a fluorescence microscope, at 200x magnification.

Real-time RT-PCR

After transfection with siRNAs, the RNA was extracted using Qiagen RNeasy kit (Qiagen, USA). Concentration was determined in a ND-1000 spectrometer (Nanodrop) and 1 μg of total RNA was converted to cDNA using a reverse-transcriptase RT enzyme (Invitrogen, USA). P-cadherin/*CDH3,* α6 Integrin/*ITGA6* and β4 Integrin/*ITGB4* TaqMan probes (Applied Biosystems, USA) were used to specifically recognize the corresponding cDNA sequences, which were amplified for 40 cycles (Applied Biosystems 7500). A TaqMan probe for *GAPDH* was also used as a housekeeping gene and relative gene expression was determined by normalization.

Co-Immunoprecipitation

BT-20 cells grown in monolayer were lysed with PBS containing 1% NP40 (Sigma-Aldrich) and 2 mM calcium chloride (Sigma-Aldrich), with phosphatase (Sigma-Aldrich) and protease inhibitors (Roche Diagnostics Gmbh). 500μg of cell lysate was precleared

with Protein G magnetic beads (Millipore, Temecula, CA) for 10 minutes at room temperature and then incubated overnight at 4ºC with 2 µg of mouse monoclonal anti-P-cadherin (Abcam, Cambridge, UK) or rabbit polyclonal anti-β4 integrin (Santa Cruz Biotechnology) or its corresponding control isotype (IgG1 mouse or IgG1 rabbit, company, respectively). The samples were then incubated with the Protein G magnetic beads (Millipore) for 10 minutes at room temperature. The beads were washed three times with washing buffer (lysis buffer diluted 1:5, containing phosphatase and protease inhibitors, as stated above) and boiled for 5 minutes in Laemmli buffer with β-mercaptoethanol (BioRad, Hercules, CA). Samples were subjected to SDS-PAGE and immunoblotting as previously described.

In vivo assay

The P-cadherin positive cell line MDA-MB-468 was used to induce tumors in immunocompromised mice. This cell line was sorted in a BD FACS Aria II, according to P-cadherin expression (R&D antibody), into two subpopulations: top 20% P-cadherin and low 20% P-cadherin fractions. The unsorted and the sorted cells were xeno-transplanted at $5x10^4$ cells (in 100 µl DMEM cell suspension) into the subcutaneous region, under the left abdominal mammary fat pad of 4-5 weeks old female N:NIH(s)II:nu/nu nude mice, using a 25-gauge needle. Mice were sacrificed after three months. Mice were maintained and housed at IPATIMUP Animal House, sited at the Medical Faculty of the University of Porto, in a pathogen-free environment, under controlled conditions of light and humidity.

Immunohistochemistry

A total of 16 tumors from 26 xenografted mice were isolated and fixed in 4% formaldehyde. Immunohistochemistry (IHC) was performed with antibodies for P-cadherin (BD Biosciences) (1:50, 1 hour, RT), α6 integrin (Sigma-Aldrich) (1:50, 1 hour, RT), β4 integrin (Santa Cruz) (1:300, 1 hour, RT), pFAK Tyr 397 (Cell Signalling) (1:50, 1 hour, RT), and pSrc Tyr 416 (Cell Signalling) (1:50, 4ºC, overnight).

High temperature (98ºC) antigen retrieval with Tris-EDTA (P-cadherin, α6 integrin, pFAK) or citrate buffer (β4 integrin, pSrc) was performed before primary antibody incubation. The primary antibodies were detected using a secondary antibody with horseradish peroxidase polymer (Cytomation Envision System HRP; DAKO, Carpinteria, CA), using diaminobenzidine (DAB) as chromogen, according to the manufacturer's instructions.

All the markers were mainly detected at the membrane of tumor cells. Concerning P-cadherin expression, all the tumors showed more than 50% of positive cells. Thus, the tumors were evaluated according with the stain intensity, being classified into P-cad high (strong stain) and P-cad low (weak and moderate stain).

According with previous published reports, the scoring for the remaining markers was considered as follows: α6 integrin stain was classified according to the stain intensity into strong (positive) and moderate/weak (negative/low) [53]; β4 integrin was classified according to the stain extension into >= 50% (positive) and <50% (negative/low) [26]; pFAK was classified according to the stain extension into >= 5% (positive) and <5% (negative/low) [54]; pSrc was considered positive when more than 50 % of tumor cells stained positive for this marker [55].

Statistical analysis

Adhesion, MFE, invasion and changes in mRNA expression levels were compared using two-tailed unpaired t-test. Immunohistochemical associations between the molecular markers were assessed by Pearson's correlation and Fisher's exact test. Statistical analyses were carried out using Prism GraphPad (La Jolla, CA) and a significant level of 5% was considered. Flow Cytometry data was analyzed with the FlowJo software package (Treestar, Ashland, OR, USA).

Competing interests

The authors indicate no competing interests.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Fundação para a Ciência e Tecnologia (FCT), Portugal, for fellowship support (A.F.V. was supported by SFRH/BPD/90303/2012, A.S.R.: SFRH/BPD/75705/2011, B.S.: SFRH/BD/69353/2010), a scientific project (PTDC/SAU-GMG/120049/2010) and for the Programa Ciência 2007 (J.P.) (POPH - QREN - Tipology 4.2). The Programme for Advanced Medical Education is sponsored by Fundação Gulbenkian, Fundação Champalimaud, Portuguese Ministry of Science, Technology and Higher Education and FCT, Portugal. IPATIMUP is an Associate Laboratory of the Portuguese Ministry of Science, Technology and Higher Education and is partially supported by FCT. R.B.C. was funded by a Breast Cancer Campaign Senior Research Fellowship.

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